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<b>(21) International Application Number:</b> PCT/US97/23706 <b>(22) International Filing Date:</b> 17 December 1997 (17.12.97)  <b>(30) Priority Data:</b> 60/033,137      20 December 1996 (20.12.96)    US 08/965,492      6 November 1997 (06.11.97)      US  <b>(71) Applicant:</b> DADE BEHRING MARBURG GMBH [DE/DE]; Emil-von-Behring-Strasse 76, D-35041 Marburg (DE).  <b>(71)(72) Applicant and Inventor:</b> ULLMAN, Edwin, F. [US/US]; 1135 Selby Lane, Atherton, CA 94027 (US).  <b>(72) Inventors:</b> LISHANSKI, Alla; Apartment 110, 840 Meridian Way, San Jose, CA 95126-3807 (US). KURN, Nurith; 2876 Ramona Street, Palo Alto, CA 94306 (US).  <b>(74) Agents:</b> RUSZALA, Lois, K. et al.; Dade Behring Inc., Legal Dept., 1717 Deerfield Road, Deerfield, IL 60015 (US).		<b>(81) Designated States:</b> CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> METHOD FOR POLYNUCLEOTIDE AMPLIFICATION  <b>(57) Abstract</b> <p>The present invention relates to a method for selectively extending an oligonucleotide primer along a specific target polynucleotide sequence in a mixture of polynucleotides. The method comprises providing the modified oligonucleotide having a 3'-end that is not extendable along any polynucleotide and extending the oligonucleotide primer selectively along the specific target polynucleotide sequence by controlling the degradation of the 3'-end of the modified oligonucleotide. In this way extension along polynucleotides other than the specific target polynucleotide sequence is substantially reduced or avoided. In another aspect the invention is an improvement in a method for amplifying a target polynucleotide sequence. The improvement comprises deriving the oligonucleotide primer from a modified oligonucleotide having a portion that hybridizes to the target polynucleotide sequence except for the 3'-end thereof, which has at least one nucleotide analog that is incapable of hybridizing to a polynucleotide. Kits for carrying out the above methods are also disclosed.</p>		

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## METHOD FOR POLYNUCLEOTIDE AMPLIFICATION

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### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention.

Significant morbidity and mortality are associated with infectious diseases. More rapid and accurate diagnostic methods are required for  
10 better monitoring and treatment of disease. Molecular methods using DNA probes, nucleic acid hybridizations and in vitro amplification techniques are promising methods offering advantages to conventional methods used for patient diagnoses.

A method for the enzymatic amplification of specific segments of  
15 DNA known as the polymerase chain reaction (PCR) method has been described. This in vitro amplification procedure is based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension by thermophilic polymerase, resulting in the exponential increase in copies of the region flanked by the primers. The different  
20 PCR primers, which anneal to opposite strands of the DNA, are positioned so that the polymerase catalyzed extension product of one primer can serve as a template strand for the other, leading to the accumulation of a discrete fragment whose length is defined by the distance between the 5' ends of the oligonucleotide primers.

25 Another method has also been described for amplifying nucleic acid sequences. This method is referred to as single primer amplification. The method provides for the amplification of a target sequence that possesses a stem-loop or inverted repeat structure where the target sequence is flanked by relatively short complementary  
30 sequences. Various methods for creating such a target sequence in

relation to the presence of a polynucleotide analyte to be detected have also been described.

The amplification methods described above require that samples suspected of having a specific nucleotide sequence be heated at about 95°C and then be repetitively thermally cycled between one or two lower temperatures and about 95°C. The higher temperatures denature duplexes and the lower temperatures permit hybridization of the primer and chain extension.

The above methods are extremely powerful techniques for high sensitivity detection of target DNA molecules present in very small amounts. The correlation between the number of original target DNA molecules and the number of specifically amplified products is influenced by a number of variables. Minor variations in buffer or temperature conditions can greatly influence reaction-to-reaction amplification efficiencies. Further, clinical samples of DNA targets can contain inhibitory factors that can suppress enzymatic amplification. In addition, such clinical samples also contain irrelevant DNA, which can be present in very large amounts relative to the target DNA molecules.

The above amplification methods suffer from interference caused by random partial hybridization of primers used in such amplification to irrelevant DNA, i.e., DNA that is not target DNA and to which the primers bind non-specifically or non-selectively. A competition between target DNA and irrelevant DNA for the enzyme and the primer thus is created. As a result the efficiency of the amplification of the target DNA molecules is decreased. At best this leads to difficulty in distinguishing amplified target DNA from amplified irrelevant DNA. The amplification of irrelevant DNA to any substantial degree can interfere with specific amplification of target DNA to prevent detection of the target DNA completely.

One approach for this problem is to avoid chain extension of low temperature non-specifically hybridized primers by heating the reaction

mixture to 95°C prior to adding a critical reagent such as a polymerase enzyme or magnesium that is required to activate the polymerase. This can be accomplished by using a wax layer to separate the various reaction components until a high temperature is reached. Alternatively,  
5 an inhibitory antibody against the polymerase can be added at low temperature. The antibody denatures at elevated temperature and allows the enzyme to become reactivated. Another approach involves the use of AmpliTaq Gold® enzyme as the polymerase in PCR reactions.

10 Another method involving chain extension of an oligonucleotide primer is a method for the detection of differences in nucleic acids described in U.S. Patent Application Serial No. 60/009,289, the disclosure of which is incorporated herein by reference. Briefly, the branch migration method detects a difference between two related nucleic acid sequences.  
15 In the method, if there is a difference between the two related nucleic acid sequences, a stable quadramolecular complex is formed comprising both of the nucleic acid sequences in double stranded form. Usually, the complex comprises a Holliday junction. Both members of at least one pair of non-complementary strands within the complex have labels. The  
20 association of the labels as part of the complex is determined as an indication of the presence of the difference between the two related sequences. The method may be employed for detecting the presence of a mutation in a target nucleic acid sequence or for detecting the presence of a target nucleic acid sequence.

25 In the above method for the detection of differences between two related DNA sequences, non-specific priming can be a problem for mutation detection by inhibition of DNA branch migration. All amplification products incorporate the "tail" sequences of the reverse primers and hence are able to participate in the formation of four-stranded DNA complexes  
30 with both specific PCR products and with each other. Since the sequences

on both sides of the junction are completely different from each other, such complexes never undergo strand separation by branch migration and thus generate non-specific signal. One approach to alleviate this problem is to use a two-step PCR procedure or nested PCR. It is highly desirable,  
5 however, to perform the above method using a single PCR reaction with just one set of primers.

A method for avoiding the above problems that is inexpensive and more controllable than the approaches mentioned above is desirable.

10 2. Description of the Related Art.

A simplified hot start PCR using AmpliTaq Gold® enzyme is discussed by Birch, *et al.*, Nature (1996) 381:445-446.

A hot start procedure using wax beads is disclosed by Chou, *et al.*, Nucleic Acids Research (1992) 20:1717-1723.

15 W. B. Barnes discusses PCR amplification of up to 35-kb DNA with high fidelity and high yield from  $\lambda$ -bacteriophage templates in Proc. Nat. Acad. Sci. USA (1994) 91:2216-2220.

A process for amplifying, detecting and/or cloning nucleic acid sequences is disclosed in U.S. Patent Nos. 4,683,195, 4,683,202,  
20 4,800,159, 4,965,188 and 5,008,182. Sequence polymerization by polymerase chain reaction is described by Saiki, *et al.*, (1986) Science, 230: 1350-1354. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase is described by Saiki, *et al.*, Science (1988) 239:487.

25 U.S. Patent No. 5,508,178 (Rose, *et al.*) describes nucleic acid amplification using a single polynucleotide primer. U.S. Patent Application Serial No. 08/140,349 filed October 20, 1993 (Laney, *et al.*), describes methods of introducing defined sequences at the 3'-end of polynucleotides. The disclosures of these references are incorporated  
30 herein by reference.

Amplification of nucleic acid sequences using oligonucleotides of random sequence as primers is described in U.S. Patent No. 5,043,272 (Hartley).

Nickel, *et al.*, J. Biol. Chem. (1992) 267:848-854 describes  
5 interactions of azidothymidine triphosphate with the cellular DNA polymerases  $\alpha$ ,  $\delta$  and  $\epsilon$  and with DNA primase.

EP 0 439 182 (Backman, *et al.*) discusses methods of amplifying target nucleic acids applicable to both polymerase and ligase chain reactions.

10

#### SUMMARY OF THE INVENTION

In its broadest aspect the present invention relates to a method for controlling the concentration of an oligonucleotide primer in a reaction medium for conducting a reaction in which the oligonucleotide primer is  
15 extended along a polynucleotide template in the presence of reagents for carrying out such extension, e.g., a nucleotide polymerase and nucleoside triphosphates. A reaction medium is provided that comprises a modified oligonucleotide together with reactants for extending the oligonucleotide primer along the polynucleotide template. The modified  
20 oligonucleotide has a degradable 3'-end that is substantially incapable of being extended along the polynucleotide template. Degradation of the 3'-end of the modified oligonucleotide results in the formation of the oligonucleotide primer. The reaction medium is subjected to controlled conditions for degrading the 3'-end of the modified oligonucleotide  
25 thereby forming the oligonucleotide primer in a controlled manner for its extension along the polynucleotide template.

Another aspect of the present invention is a method for selectively extending an oligonucleotide primer along a specific target polynucleotide sequence in a mixture of polynucleotides. The method  
30 comprises providing in combination a mixture of polynucleotides and a

modified oligonucleotide having a 3'-end that is not extendable along any polynucleotide and extending degraded modified oligonucleotide along the specific target polynucleotide sequence by controlling the degradation of the 3'-end of the modified oligonucleotide to give  
5 degraded modified oligonucleotide, which serves as an oligonucleotide primer in a chain extension reaction. In this way extension of the oligonucleotide primer along polynucleotides other than the specific target polynucleotide sequence is substantially reduced or avoided.

In another aspect the present invention provides an improvement  
10 in a method for amplifying a target polynucleotide sequence. The method comprises combining the target polynucleotide sequence with reagents for amplifying the target polynucleotide sequence and subjecting the combination to conditions wherein the target polynucleotide sequence is amplified. The reagents comprise a modified  
15 oligonucleotide and a polymerase. The improvement comprises the modified oligonucleotide having a portion that hybridizes to the target polynucleotide sequence except for the 3'-end thereof, which has at least one nucleotide analog that is incapable of hybridizing to a polynucleotide.

20 Another embodiment of the present invention is a method for amplifying a target polynucleotide sequence. A combination is provided that comprises the target polynucleotide sequence and reagents for conducting an amplification of the target polynucleotide sequence. The reagents comprise a polymerase, nucleoside triphosphates and a modified  
25 oligonucleotide, which comprises at least one nucleotide analog at the 3'-end thereof. The nucleotide analog is substantially non-hybridizable to any polynucleotide sequence and is also removable by a 3' to 5' exonuclease at a rate that is slower than for a natural nucleotide. The combination is subjected to conditions for amplifying the target polynucleotide sequence  
30 wherein the nucleotide analog is removed from the modified



oligonucleotide. Extension of the oligonucleotide primer along the target polynucleotide sequence occurs to produce an extended oligonucleotide primer.

Another aspect of the present invention is a method for amplifying  
5 a target polynucleotide sequence in which the target polynucleotide  
sequence is combined with reagents for amplifying the target  
polynucleotide sequence and the combination is subjected to conditions  
to amplify the target polynucleotide sequence. The reagents comprise  
an oligonucleotide primer and a polymerase. The improvement  
10 comprises using a modified oligonucleotide having a portion that  
hybridizes to the target polynucleotide sequence except for the 3'-end  
thereof. The 3'-end is degradable at a rate of degradation that is slower  
for the modified oligonucleotide than for a corresponding oligonucleotide  
that does contain a modified nucleotide.

15 Another aspect of the present invention is an improvement in a  
method for forming multiple copies of a target polynucleotide sequence.  
A first oligonucleotide primer ("first primer") is hybridized to the 3'-end of  
the target sequence. The first primer is extended along at least the  
target sequence in the presence of a polymerase. The first primer is  
20 capable of hybridizing to, and being extended along extended first  
primer or an extended second oligonucleotide primer ("second primer")  
that is different from the first primer. The extended second primer  
results from the extension of a second primer capable of hybridizing to  
and extending along a polynucleotide that is complementary  
25 (complementary polynucleotide) to the target sequence. Extended first  
primer is dissociated from the target sequence. The first or second  
primer is hybridized to the 3'-end of the extended first primer. The first or  
second primer is extended along the extended first primer and extended  
first primer or extended second primer is dissociated from extended first  
30 primer. The first primer is hybridized to the 3'-end of the extended first or

second primer. Steps (e)-(g) are repeated. The improvement comprises using a modified oligonucleotide in place of the first oligonucleotide primer. The modified oligonucleotide has a portion that hybridizes to the target polynucleotide sequence except for the 3'-end thereof, which 3'-  
5 end has at least one nucleotide analog that is incapable of hybridizing to a polynucleotide.

Another embodiment of the present invention is a kit comprising in packaged combination (a) a modified oligonucleotide comprising at least one nucleotide analog at the 3'-end thereof, the nucleotide analog being  
10 substantially non-hybridizable to any normal nucleotide in a polynucleotide sequence, and (c) a 3' to 5' exonuclease. In one embodiment the nucleotide analog is removable by a 3' to 5' exonuclease at a rate that is slower than for a natural nucleotide, (b) nucleoside triphosphates

15

#### Brief Description of the Drawings

Figs. 1 is a schematic diagram depicting an embodiment in accordance with the present invention.

Fig. 2 is a schematic diagram depicting an alternate embodiment  
20 in accordance with the present invention.

Fig. 3 is a schematic diagram depicting an alternate embodiment in accordance with the present invention.

Fig. 4 is a schematic diagram depicting an alternate embodiment in accordance with the present invention.

25

#### Description of the Specific Embodiments

In the present invention an amplification of a target polynucleotide sequence is conducted using a modified oligonucleotide having one or more nucleotides at the 3'-end that are modified to render them  
30 unnatural resulting in inhibition of their ability to hybridize to natural

nucleotides in DNA or in inhibition of chain extension by other means. The modified oligonucleotides used are not extendable on either the desired complementary target polynucleotide sequence or on irrelevant DNA, to which the primer may non-specifically bind. When all of the  
5 amplification reagents are mixed with the sample, extension of the primer along any polynucleotide present in the mixture is inhibited because of the presence of at least one unnatural nucleotide at the 3'-end. Modifications are chosen so as to obtain modified nucleotides that are relatively resistant to 3'-exonuclease activity but undergo slow  
10 degradation to remove the modified nucleotides as the temperature is increased. Upon removal of the modified nucleotides, the degraded modified oligonucleotide is an oligonucleotide primer that undergoes chain extension along the target polynucleotide sequence. The background products resulting from amplification of irrelevant DNA are  
15 greatly decreased because hybridization leading to chain extension only takes place at an elevated temperature where binding is relatively selective.

In essence, the control of the amplification in accordance with the present invention is achieved as a result of controlling the *in situ*  
20 concentration of the oligonucleotide primer. This is accomplished by utilizing a modified oligonucleotide having a degradable 3'-end that is substantially incapable of being extended along, or inefficiently extendable along, the target polynucleotide sequence or polynucleotide template. Degradation of the 3'-end of the modified oligonucleotide  
25 results in the formation of the oligonucleotide primer. The reaction medium is subjected to controlled conditions for degrading the 3'-end of the modified oligonucleotide thereby forming the oligonucleotide primer in a controlled manner for its extension along the polynucleotide template.

The present method has application to a number of procedures where amplification of a target polynucleotide sequence is carried out using thermal cycling. The use of the present method eliminates the use of nested primers or other means that were previously required to provide sufficiently low background for an amplification method to provide a meaningful result.

Before proceeding further with a description of the specific embodiments of the present invention, a number of terms will be defined.

Chain extension of an oligonucleotide -- extension of an oligonucleotide along a polynucleotide template (chain of nucleotides) to produce a chain extension product that is the complement of the polynucleotide template. In the context of an amplification chain extension usually involves temperature cycling, i.e., elevating the temperature of the reaction mixture to cause hybridized polynucleotide sequences to denature, cooling the reaction mixture to permit binding of an oligonucleotide primer to its respective target polynucleotide sequence and subsequent extension along the target polynucleotide sequence, elevating the temperature of the reaction medium to cause hybridized polynucleotide sequences to denature, and repeating the above. In general, in-primer extension amplification primers hybridize to, and are extended along (chain extended along), at least the target sequence within the target polynucleotide and, thus, the target sequence acts as a template. The extended primers are "chain extension products." Reagents for carrying out a chain extension of an oligonucleotide primer along a polynucleotide template include a nucleotide polymerase and nucleoside triphosphates.

One important method utilizing chain extension of an oligonucleotide primer is that for the amplification of nucleic acids or polynucleotides, such as a target polynucleotide sequence. Such methods generally result in the formation of one or more copies of a

nucleic acid or polynucleotide molecule or in the formation of one or more copies of the complement of a nucleic acid or polynucleotide molecule, usually a target polynucleotide sequence, present in a medium.

5           One such method for the enzymatic amplification of specific double stranded sequences of DNA is known as the polymerase chain reaction (PCR), as described above. This *in vitro* amplification procedure is based on repeated cycles of denaturation, annealing of at least two different oligonucleotide primers, and primer extension, i.e.,  
10   "chain extension" of such primers, by thermophilic template dependent polynucleotide polymerase, resulting in the exponential increase in copies, i.e., "chain extension products of the above primers," of the target polynucleotide sequence flanked by the primers. The two different PCR primers, which anneal to opposite strands of the DNA, are  
15   positioned so that the polymerase catalyzed extension product of one primer can serve as a template strand for the other, leading to the accumulation of a discrete double stranded fragment whose length is defined by the distance between the 5' ends of the oligonucleotide primers.

20           Another method for amplification is mentioned above and involves amplification of a single stranded polynucleotide using a single polynucleotide primer. The single stranded polynucleotide that is to be amplified contains two non-contiguous sequences that are complementary to one another and, thus, are capable of hybridizing  
25   together to form a stem-loop structure. This single stranded polynucleotide may be already part of a target polynucleotide sequence or may be created as the result of the presence of a target polynucleotide sequence.

          Another method involving chain extension of an oligonucleotide  
30   primer is a method for the detection of differences in nucleic acids

described in U.S. Patent Application Serial No. 60/009,289, the disclosure of which is incorporated herein by reference above. Generally, in the method a medium suspected of containing two related nucleic acid sequences is treated to provide two partial duplexes each comprised of fully matched duplexes having at one end non-complementary end portions. The partial duplexes are related in that, except for the difference, one of the strands S1 of one of the partial duplexes is complementary to one of the strands S1' of the other of the partial duplexes and the other of the strands S2 of one of the partial duplexes is complementary to the other of the strands S2' of the other of the partial duplexes. The medium is subjected to conditions that permit the binding of S1 to S1' and S2 to S2', respectively. If the medium contains a difference between the related nucleic acid sequences, a stable complex is formed comprising strands S1, S1', S2 and S2'. A determination is made whether the stable complex is formed, the presence thereof indicating the presence of a difference between the related nucleic acid sequences.

Nucleotide -- a base-sugar-phosphate combination that is the monomeric unit of nucleic acid polymers, i.e., DNA and RNA.

Natural nucleotide -- a nucleotide generally found in nature; such natural nucleotides include bases such as adenine, uridine, cytidine, thymidine, guanine and so forth.

Modified nucleotide -- is the unit in a modified oligonucleotide that differs from a natural nucleotide by some modification. The modified nucleotide is unable to hybridize to any natural nucleotide and/or to be extended by a polymerase along a template. The nature of the modified nucleotide for purposes of the present invention is described in more detail below in the definition of modified oligonucleotide.

Nucleoside -- is a base-sugar combination or a nucleotide lacking a phosphate moiety.

Target polynucleotide sequence -- a sequence of nucleotides to be identified, usually existing within a portion (target polynucleotide) or all of a polynucleotide analyte, the identity of which is known to an extent sufficient to allow preparation of various primers and other molecules necessary for conducting an amplification of the target sequence contained within the target polynucleotide. In general, in primer extension amplification primers hybridize to, and are extended along (chain extended along), at least the target sequence within the target polynucleotide and, thus, the target sequence acts as a template. The extended primers are "chain extension products." The target sequence usually lies between two defined sequences but need not. In general, the primers hybridize with the defined sequences or with at least a portion of such target polynucleotide, usually at least a ten nucleotide segment at the 3'-end thereof and preferably at least 15, frequently 20 to 50, nucleotide segment thereof. The target polynucleotide sequence usually contains from about 30 to 5,000 or more nucleotides, preferably 50 to 1,000 nucleotides. The target polynucleotide is generally a fraction of a larger molecule or it may be substantially the entire molecule. The minimum number of nucleotides in the target polynucleotide sequence is selected to assure that the presence of target polynucleotide in a sample can be determined. Very roughly, the sequence length is usually greater than about  $1.6 \log L$  nucleotides where L is the number of base pairs in the genome of the biologic source of the sample. The maximum number of nucleotides in the target polynucleotide is normally governed by the length of the polynucleotide analyte and its tendency to be broken by shearing, or other processes during isolation and any procedures required to prepare the sample for assay and the efficiency of detection and/or amplification of the sequence.

Oligonucleotide -- a polynucleotide, usually single stranded, usually a synthetic polynucleotide but may be a naturally occurring

polynucleotide. The oligonucleotide(s) are usually comprised of a sequence of 5 to 150 or more nucleotides, preferably, 10 to 100 nucleotides, more preferably, 15 to 50 nucleotides in length.

Various well-known techniques can be employed for preparing oligonucleotides. Such sequences can be obtained by biological synthesis or by chemical synthesis. For short sequences (up to about 100 nucleotides) chemical synthesis is frequently more economical as compared to biological synthesis. For longer sequences standard replication methods employed in molecular biology can be used such as the use of M13 for single stranded DNA as described by J. Messing, Methods Enzymol(1983) 101: 20-78.

In addition to standard cloning techniques, in vitro enzymatic methods may be used such as polymerase catalyzed reactions. For preparation of RNA, T7 RNA polymerase and a suitable DNA template can be used. For DNA, polymerase chain reaction (PCR) and single primer amplification are convenient.

Other chemical methods of polynucleotide or oligonucleotide synthesis include phosphotriester and phosphodiester methods (Narang, et al., Meth. Enzymol (1979) 68: 90) and synthesis on a support (Beaucage, et al., Tetrahedron (1981) Letters 22: 1859-1862) as well as phosphoramidate technique, Caruthers, M. H., et al., "Methods in Enzymology," Vol. 154, pp. 287-314 (1988), and others described in "Synthesis and Applications of DNA and RNA," S.A. Narang, editor, Academic Press, New York, 1987, and the references contained therein.

End of an oligonucleotide -- as used herein this phrase refers to nucleotides, including the terminal nucleotide, at either the 3'- or 5'-opposing sides of an oligonucleotide.

Terminus of an oligonucleotide -- as used herein this term refers to the terminal nucleotide at either the 3'- or 5'- end of an oligonucleotide.



Oligonucleotide primer(s) -- an oligonucleotide that is usually employed in a chain extension on a polynucleotide template such as in, for example, an amplification of a nucleic acid. The oligonucleotide primer is usually a synthetic deoxynucleotide that is single stranded, containing a sequence at its 3'-end that is capable of hybridizing with a defined sequence of the target polynucleotide. Normally, an oligonucleotide primer, and particularly its 3'-end, has preferably 70%, more preferably 90%, most preferably 100%, complementarity to the defined sequence. The number of nucleotides in the hybridizable sequence of a oligonucleotide primer, which hybridizes to a target polynucleotide sequence, should be such that stringency conditions used to hybridize the oligonucleotide primer will prevent excessive random non-specific hybridization. The number of nucleotides in the oligonucleotide primer will be the same as the defined sequence of the target polynucleotide to which it binds, namely, at least 12 nucleotides, preferably, at least 15 nucleotides, and generally from about 12 to 50, preferably, 15 to 30, nucleotides.

Modified oligonucleotide - an oligonucleotide that possess one or more nucleotides having a chemical modification ("modified nucleotide" sometimes referred to herein as a "nucleotide analog") at the 3'-end thereof as compared to an oligonucleotide having a natural or unmodified nucleotide at its 3'-end. For the purpose of defining the position of a modified nucleotide, 3'-end includes any of the last three ribophosphates at the 3'-terminus of the oligonucleotide, usually any of the last two ribonucleotides. The modified nucleotide is unable to hybridize to any substantial degree to any natural nucleotide and, thus, the modified oligonucleotide is unable to chain extend, i.e., not extendable, along a polynucleotide to which it is hybridized. Accordingly, chain extension does not occur to any substantial degree unless and until the modification or the modified nucleotide is removed. The

modified nucleotide(s) render the 3'-end of the modified oligonucleotide substantially incapable of hybridizing with a target polynucleotide sequence. While it is desirable in the present invention that the 3'-end be incapable of hybridizing to the target polynucleotide sequence, a  
5 small degree of hybridization, e.g., 1 to 5%, may be tolerated consistent with the aim of the present invention, namely, to substantially reduce the amount of background products in an amplification of a target polynucleotide sequence. Such a degree of tolerated hybridization may result in inefficient extension of the modified oligonucleotide along the  
10 target polynucleotide sequence and in that sense the modified oligonucleotide may be inefficiently extendable.

The modification provides for controlled degradation of the 3'-end portion of the modified oligonucleotide when the modified oligonucleotide is not hybridized to a target polynucleotide sequence. Modified  
15 nucleotides for the modified oligonucleotide are selected that reduce the rate of degradation and subsequent chain extension of the modified oligonucleotide by at least 20%, preferably, by at least 50 to 98%, seldom greater than 99%, the modified oligonucleotide being capable of storage with a polymerase, target polynucleotide and nucleoside  
20 triphosphates preferably for at least 5 minutes, more preferably, at least 10 minutes, at room temperature without substantial chain extension. It is also desirable that the 3'-end of the modified oligonucleotide be degradable no more rapidly and preferably 2 to 100 times slower when the modified oligonucleotide is hybridized to a polynucleotide sequence  
25 relative to when it is not hybridized.

The modified oligonucleotide has at least one modified nucleotide, preferably a contiguous sequence of 2 to 5 modified nucleotides terminating at the 3'-end of the primer. The modified nucleotides are usually attached directly to a sequence ("priming sequence") of about 12  
30 to 50 nucleotides, preferably, about 15 to 30 nucleotides that are

hybridizable with the intended "priming site" of the target polynucleotide sequence, i.e., the site to which degraded modified oligonucleotide is to bind for chain extension. Accordingly, such sequence of nucleotides is at least 80%, preferably, 90%, more preferably, 100%, complementary to the intended priming site. The 5'-end of the priming sequence may have a sequence of nucleotides, including modified nucleotides, that are not hybridizable with, or not complementary to, the priming site of the target polynucleotide sequence.

Any modification that accomplishes the purposes of the present invention may be utilized. The modification should be one that is degradable under the conditions of the reaction in which the modified oligonucleotide is used. On the other hand, the modification should not degrade so fast that one cannot obtain the control of degradation necessary to achieve the benefits of the present invention. The type and extent of modification of the 3'-end of the modified oligonucleotide is generally determined empirically with the goal of achieving the above parameters for control of degradation of the 3'-end of the modified oligonucleotide. For example, in determining the type and extent of modification subject to degradation by a 3' to 5' exonuclease, one can utilize a system in which the modified oligonucleotide is employed to generate an oligonucleotide primer for use as a forward primer in a reaction system mimicking a PCR amplification. Briefly, the modified oligonucleotide is combined in an appropriate medium for conducting PCR, which contains buffer, magnesium and a 3' to 5' exonuclease but not a target polynucleotide sequence. The reaction medium is subjected to thermal cycling for a period of time. Then, the modified oligonucleotide is used a forward primer in a PCR amplification in which a target polynucleotide sequence is present in the reaction medium. The extent of delay of the accumulation of an amplicon (chain extended oligonucleotide primer) is determined. This approach is described in

more detail in Example 1 below, which is by way of illustration and not limitation.

One example of a modified nucleotide in a modified oligonucleotide in accordance with the present invention is a nucleotide  
5 that is substantially incapable of hydrogen bonding.

In one embodiment the modified nucleotide is a natural nucleotide that has a 3'-hydroxyl group that has been modified such as by formation of an ester, amide, sulfate or glycoside and thus is not chain extendable. Preferably, such a modified nucleotide is heat or light labile  
10 and thus the modified nucleotide is removable as the temperature of the reaction medium is raised or the medium is irradiated as the case may be. In another approach such a modified nucleotide may be removed enzymatically. Other methods of removal of such a modified nucleotide will be suggested to those skilled in the art in view of the above  
15 disclosure. For example, where the modification is an ester, removal is achieved in accordance with the present invention by use of an enzyme that is a thermally stable esterase. Alternatively, where a glycoside of the 3'-hydroxyl group is employed, the glycosidic linkage is cleaved by a thermally stable glycosidase. For example, a  $\beta$ -galactosyl group can be  
20 attached to the 3'-end of a modified oligonucleotide and a thermally stable  $\beta$ -galactosidase can be used in the reaction medium.

In another embodiment, the modification is selected such that the modified nucleotide or nucleotides are removed by an enzyme having 3'-exonuclease activity when the modified oligonucleotide is not bound to a  
25 sequence to which it can hybridize. One factor in the selection of the modified nucleotides in this approach is the specificity of the polymerase used in an amplification. The particular modified oligonucleotide chosen is one having one or more modified nucleotides at its 3'-end, which are then subjected to degradation by heating with the enzyme having 3'-  
30 exonuclease activity to be used. The rate of degradation can be readily

followed by gel electrophoresis. Preferably, the rate should be slower than degradation of an unmodified oligonucleotide that is similar to the modified oligonucleotide but does not possess the modified nucleotides.

Where the rate is particularly slow, it will usually be desirable to  
5 include one or more groups in the complementary portion of the modified oligonucleotide that inhibit further degradation. This can be done, for example, by including phosphorothioates between the 3'-end of the modified oligonucleotide and the penultimate nucleotide of the portion of  
10 the modified oligonucleotide that is complementary to the target polynucleotide sequence. Optionally, phosphorothioates may be introduced at other sites near the 3'-end as well. These modified oligonucleotides, when subjected to 3'-exo<sup>+</sup>-polymerase catalyzed degradation in accordance with the present invention, will be resistant to degradation past the phosphorothioate groups.

15 Chemical modifications of a natural nucleotide to produce an unnatural or modified nucleotide are described hereinbelow by way of example and not limitation. Ethenoadenosine has an ethylene bridge between the 6-amino group and the ring nitrogen at position 1 that blocks any possible hydrogen bonding. Other modifications include  
20 alkylation at the 6-oxygen of guanine, the 4-oxygen of thymine, the ring nitrogens at the 5-position of the purines, or the 3-positions of the pyrimidines, or the removal of the 2-amino group of guanine or the 4-amino group of cytosine. Heterocyclic groups other than purines and pyrimidines can also be used. In that regard it is preferable to use  
25 derivatives that can be purchased in a form convenient for solid state synthesis of the modified oligonucleotide, usually as phosphorimidates. Other heterocycles include, for example, triazine, unsubstituted pyrimidine, pyridines, deazapurines, pyridopyrroles and the like. The particular structure of the modified nucleotide is not critical so long as the

enzyme can remove it when it is not hybridized and so long as it does not support chain extension.

Another example, by way of illustration and not limitation, of a suitable modification is a nucleotide that is modified on the ribose.

5 Ribonucleotides are candidates because oligonucleotides terminating in ribonucleotides cannot be extended by most polymerases. When ribonucleotides are employed, an enzyme must be included that can exolytically remove the ribonucleotide from the modified oligonucleotide when the modified oligonucleotide is not hybridized to a complementary  
10 strand and cannot readily remove the ribonucleotide when the primer is hybridized. Other examples of modification of the ribose include 3'-deoxy derivatives including those in which the 3'-hydroxy is replaced by a functionality other than hydrogen such as an azide group.

Many modified nucleotides and oligonucleotides containing such  
15 modified nucleotides are commercially available or known in the literature. For example, etheno-deoxy A, O-6-methyl deoxy G and O-4-methyl deoxy T are commercially available from Oligos Etc., Wilsonville, Oregon. Non-hydrogen bonding nucleosides are discussed by Moran, *et al.*, in Nucleic Acids Research (1996) 24(11):2044-2052 and include 4-  
20 methylindole  $\beta$ -nucleoside,  $\alpha$ -naphthalene nucleoside,  $\alpha$ -pyrene nucleoside, and the like. N3-( $\beta$ -D-ribofuranoside derivatives such as 4-amino-1-(2'-deoxy- $\beta$ -D-ribofuranosyl)-2(1H)-pyridinone and oligonucleotides comprising such modified nucleotides are disclosed by Charczuk, *et al.*, in Helv. Chim. Acta (1987) 70(3):717-725. Huang, *et al.*, discuss arabinosylcytosine 5'-triphosphate and other modified  
25 nucleosides in Cancer Res (1991) 51:6110-6117. Solomon, *et al.*, disclose C-linked deoxyribosides of 2-hydroxypyridine and 2-hydroxyquinoline in Tetrahedron Letters (1991) 32(28):3297-3300; see also Solomon, *et al.*, J. Org. Chem. (1993) 58:2232-2243. Other

modified nucleosides and modified oligonucleotides may be synthesized by employing well-known synthetic techniques.

The chemical modification can be introduced into the oligonucleotide to be modified by various well-known techniques as described above for the preparation of oligonucleotides in general. Both biological synthesis or chemical synthesis can be employed. In one approach phosphotriester and phosphodiester methods can be used (Narang, et al., Meth. Enzymol (1979) 68: 90) and synthesis on a support (Beaucage, et al., Tetrahedron (1981) Letters 22: 1859-1862) as well as phosphoramidate technique, Caruthers, M. H., et al., "Methods in Enzymology," Vol. 154, pp. 287-314 (1988), and others described in "Synthesis and Applications of DNA and RNA," S.A. Narang, editor, Academic Press, New York, 1987, and the references contained therein. Controlled pore glass having a modified nucleotide bound to the surface is available for solid phase DNA synthesis employing the phosphoramidate technique. Accordingly, both automated and manual synthesis can be carried out. Modified oligonucleotides containing more than one modified nucleotide can be prepared in a similar manner by adding a modified nucleotide that has a 3'-hydroxyl to which another modified nucleotide can be added and repeating this process.

In addition to standard cloning techniques, in vitro enzymatic methods may be used such as polymerase catalyzed reactions. For preparation of RNA, T7 RNA polymerase and a suitable DNA template can be used. For DNA, polymerase chain reaction (PCR) and single primer amplification are convenient.

In another approach the 3'-hydroxyl group of a natural nucleotide may be derivatized by adding a single modified nucleotide in solution phase.

Some of the references cited above disclosing modified nucleosides that can be used in the present invention also describe syntheses of oligonucleotides

- containing the modified nucleotides. See, for example, Solomon, *et al.*, J. Org. Chem. (1993) 58:2232-2243 and Charczuk, *et al.*, in Helv. Chim. Acta (1987) 70(3):717-725.

- Phosphorothioate -- a nucleotide monophosphate in which an oxygen of at least one phosphate has been replaced by sulfur. An oxygen of 1 to 5 phosphates may be replaced by sulfur, more preferably, the oxygen of 1 to 2 phosphates is replaced. The sulfur is frequently bound solely to phosphorus (phosphorothioate group), but can also be bound to a ribose carbon atom or carbon atom of a label. Thus, such modified oligonucleotides contain at least one, preferably 1 to 5, more preferably, 1 to 2, phosphorus-sulfur bonds. These sulfur-containing modified oligonucleotides can be prepared according to known techniques. See, for example, WO9008838, WO8911486, U.S. Patent No. 4,910,300, EP318245, the relevant disclosures of which are incorporated herein by reference. Other methods of preparing a phosphorothioate containing polynucleotide are described by (a) Yau, *et al.*, Tetrahedron Lett. (1990)31(14): 1953-1956; (b) Brill, *et al.*, *ibid.* (1989) 30(48):6621-6624; (c) Caruthers, *et al.*, Nucleic Acids Symp. Ser. (1989)21: 119-120; (d) Caruthers, *et al.*, Nucleosides Nucleotides (1988)8(5-6): 1011-1014; (e) Brill, *et al.*, J. Am. Chem. Soc. (1989)111(6): 2321-2322.

- Nucleoside triphosphates -- nucleosides having a 5'-triphosphate substituent. The nucleosides are pentose sugar derivatives of nitrogenous bases of either purine or pyrimidine derivation, covalently bonded to the 1'-carbon of the pentose sugar, which is usually a deoxyribose or a ribose. The purine bases include adenine(A), guanine(G), inosine, and derivatives and analogs thereof. The pyrimidine bases include cytosine (C), thymine (T), uracil (U), and derivatives and analogs thereof.



Nucleoside triphosphates include deoxyribonucleoside triphosphates such as dATP, dCTP, dGTP and dTTP and ribonucleoside triphosphates such as rATP, rCTP, rGTP and rUTP. The term "nucleoside triphosphates" also includes derivatives and analogs thereof, which are exemplified by those derivatives that are recognized and polymerized in a similar manner to the underivitized nucleoside triphosphates. Examples of such derivatives or analogs, by way of illustration and not limitation, are those which are modified with a reporter group, biotinylated, amine modified, radiolabeled, alkylated, and the like and also include phosphorothioate, phosphite, ring atom modified derivatives, and the like. The reporter group can be a fluorescent group such as fluorescein, a chemiluminescent group such as luminol, a terbium chelator such as N-(hydroxyethyl) ethylenediaminetriacetic acid that is capable of detection by delayed fluorescence, and the like. The term "nucleoside triphosphate" includes the derivatives and analogs thereof.

Nucleotide polymerase – a catalyst, usually an enzyme, for forming an extension of an oligonucleotide along a DNA template where the extension is complementary to the template. The nucleotide polymerase is a template dependent polynucleotide polymerase and utilizes nucleoside triphosphates as building blocks for extending the 3'-end of a oligonucleotide to provide a sequence complementary with the single stranded portion of the polynucleotide to which the oligonucleotide is hybridized to form a duplex. Usually, the catalysts are enzymes, such as DNA polymerases.

3' to 5' exonuclease – for purposes of the present invention an enzyme is considered to be a 3' to 5' exonuclease, or to have 3' to 5' exonuclease activity, when, under the conditions of the reactions contemplated herein, it catalyzes the removal or cleavage of nucleotides from the 3'-end of a modified oligonucleotide when such modified oligonucleotide is not hybridized to a target polynucleotide sequence and

may also act as a nucleotide polymerase (in the latter sense it may be considered as a polymerase comprising a 3' to 5' exonuclease). The enzyme cleaves nucleotides of the oligonucleotide primer at least up to and including the modified nucleotides. At such point the degraded  
5 modified oligonucleotide is extendable at its 3'-terminus and can act as an oligonucleotide primer when hybridized to the target polynucleotide sequence. The 3' to 5'-exonucleases useful in the present invention must be stable under the conditions used in the present method and are usually thermally stable nucleotide polymerases.

10 Such enzymes may be derived from any source such as cells, bacteria, such as E. coli, plants, animals, virus, thermophilic bacteria, and so forth wherein the polymerase may be modified chemically or through genetic engineering to provide for thermal stability and/or increased activity. Such enzymes include Pfu DNA polymerase  
15 (native and recombinant) from Stratagene, La Jolla, CA, Ultma DNA polymerase from Perkin Elmer, Foster City, CA, r Bst DNA polymerase from Epicentre Technologies, Madison, WI, VENT DNA polymerase from New England Biolabs, Beverly, MA, Tli DNA polymerase from Promega Corp., Madison, WI, and Pwo DNA polymerase from Boehringer  
20 Mannheim, Indianapolis, IN, and the like.

Polynucleotide analyte--a compound or composition to be measured in an assay; a polymeric nucleotide, which in the intact natural state can have about 20 to 500,000 or more nucleotides and in an isolated state can have about 30 to 50,000 or more nucleotides, usually  
25 about 100 to 20,000 nucleotides, more frequently 500 to 10,000 nucleotides. It is thus obvious that isolation of the analyte from the natural state often results in fragmentation. The polynucleotide analytes include nucleic acids from any source in purified or unpurified form including DNA (dsDNA and ssDNA) and RNA, including t-RNA, m-RNA,  
30 r-RNA, mitochondrial DNA and RNA, chloroplast DNA and RNA,

DNA-RNA hybrids, or mixtures thereof, genes, chromosomes, plasmids, the genomes of biological material such as microorganisms, e.g., bacteria, yeasts, viruses, viroids, molds, fungi, plants, animals, humans, and fragments thereof, and the like. The polynucleotide analyte can be  
5 only a minor fraction of a complex mixture such as a biological sample. The analyte can be obtained from various biological materials by procedures well known in the art. Some examples of such biological materials by way of illustration and not limitation are disclosed in U.S. Patent No. 5,508,178 (Rose, *et al.*), the relevant portions of which are  
10 incorporated herein by reference.

Wholly or partially sequentially – when reagents utilized in the present invention are combined other than concomitantly (simultaneously), one or more may be combined with one or more of the remaining reagents to form a subcombination. Each subcombination  
15 can then be subjected to one or more steps of the present method. Thus, each of the subcombinations can be incubated under conditions to achieve one or more of the desired results.

Hybridization (hybridizing) and binding – in the context of nucleotide sequences these terms are used interchangeably herein.  
20 The ability of two nucleotide sequences to hybridize with each other is based on the degree of complementarity of the two nucleotide sequences, which in turn is based on the fraction of matched complementary nucleotide pairs. The more nucleotides in a given sequence that are complementary to another sequence, the more  
25 stringent the conditions can be for hybridization and the more specific will be the binding of the two sequences. Increased stringency is achieved by elevating the temperature, increasing the ratio of cosolvents, lowering the salt concentration, and the like.

Homologous or substantially identical – In general, two  
30 polynucleotide sequences that are identical or can each hybridize to the

same polynucleotide sequence are homologous. The two sequences are homologous or substantially identical where the sequences each have at least 90%, preferably 100%, of the same or analogous base sequence where thymine (T) and uracil (U) are considered the same.

- 5 Thus, the ribonucleotides A, U, C and G are taken as analogous to the deoxynucleotides dA, dT, dC, and dG, respectively. Homologous sequences can both be DNA or one can be DNA and the other RNA.

Complementary -- Two sequences are complementary when the sequence of one can bind to the sequence of the other in an anti-parallel  
10 sense wherein the 3'-end of each sequence binds to the 5'-end of the other sequence and each A, T(U), G, and C of one sequence is then aligned with a T(U), A, C, and G, respectively, of the other sequence.

Copy of a sequence -- a sequence that is a direct identical copy of a single stranded polynucleotide sequence as differentiated from a  
15 sequence that is complementary to the sequence of such single stranded polynucleotide.

Member of a specific binding pair ("sbp member") -- one of two different molecules, having an area on the surface or in a cavity which specifically binds to and is thereby defined as complementary with a  
20 particular spatial and polar organization of the other molecule. The members of the specific binding pair are referred to as ligand and receptor (antiligand). These may be members of an immunological pair such as antigen-antibody, or may be operator-repressor, nuclease-nucleotide, biotin-avidin, hormones-hormone receptors, nucleic  
25 acid duplexes, IgG-protein A, DNA-DNA, DNA-RNA, and the like.

Ligand -- any compound for which a receptor naturally exists or can be prepared.

Receptor ("antiligand") -- any compound or composition capable of recognizing a particular spatial and polar organization of a molecule,  
30 e.g., epitopic or determinant site. Illustrative receptors include naturally

occurring receptors, e.g., thyroxine binding globulin, antibodies, enzymes, Fab fragments, lectins, nucleic acids, repressors, protection enzymes, protein A, complement component C1q, DNA binding proteins or ligands and the like.

- 5           Small organic molecule -- a compound of molecular weight less than 1500, preferably 100 to 1000, more preferably 300 to 600 such as biotin, fluorescein, rhodamine and other dyes, tetracycline and other protein binding molecules, and haptens, etc. The small organic molecule can provide a means for attachment of a nucleotide sequence
- 10   to a label or to a support.

- Support or surface -- a porous or non-porous water insoluble material. The support can be hydrophilic or capable of being rendered hydrophilic and includes inorganic powders such as silica, magnesium sulfate, and alumina; natural polymeric materials, particularly cellulosic
- 15   materials and materials derived from cellulose, such as fiber containing papers, e.g., filter paper, chromatographic paper, etc.; synthetic or modified naturally occurring polymers, such as nitrocellulose, cellulose acetate, poly (vinyl chloride), polyacrylamide, cross linked dextran, agarose, polyacrylate, polyethylene, polypropylene,
- 20   poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), nylon, poly(vinyl butyrate), etc.; either used by themselves or in conjunction with other materials; glass available as Bioglass, ceramics, metals, and the like. Natural or synthetic assemblies such as liposomes, phospholipid vesicles, and cells can also be
- 25   employed.

Binding of sbp members to a support or surface may be accomplished by well-known techniques, commonly available in the literature. See, for example, "Immobilized Enzymes," Ichiro Chibata, Halsted Press, New York (1978) and Cuatrecasas, J. Biol. Chem.,

245:3059 (1970). The surface can have any one of a number of shapes, such as strip, rod, particle, including bead, and the like.

Label or reporter group or reporter molecule -- a member of the signal producing system. Usually the label or reporter group or molecule  
5 is conjugated to or becomes bound to a polynucleotide probe or an oligonucleotide primer and is capable of being detected directly or, through a specific binding reaction, and can produce a detectable signal. Labels include a polynucleotide primer or specific polynucleotide sequence that can provide a template for amplification or ligation or act  
10 as a ligand such as for a repressor protein. Preferably, an oligonucleotide primer will have, or be capable of having, a label. In general, any label that is detectable can be used. The label can be isotopic or nonisotopic, usually non-isotopic, and can be a catalyst, such as an enzyme, a polynucleotide coding for a catalyst, promoter, dye,  
15 fluorescent molecule, chemiluminescer, coenzyme, enzyme substrate, radioactive group, a small organic molecule, amplifiable polynucleotide sequence, a particle such as latex or carbon particle, metal sol, crystallite, liposome, cell, etc., which may or may not be further labeled with a dye, catalyst or other detectable group, and the like. The label is  
20 a member of a signal producing system and can generate a detectable signal either alone or together with other members of the signal producing system. The label can be bound directly to a nucleotide sequence or can become bound thereto by being bound to an sbp member complementary to an sbp member that is bound to a nucleotide  
25 sequence.

Signal producing system -- The signal producing system may have one or more components, at least one component being the label or reporter group. The signal producing system generates a signal that relates to the presence or amount of target polynucleotide sequence or a  
30 polynucleotide analyte in a sample. The signal producing system

includes all of the reagents required to produce a measurable signal. When the label is not conjugated to a nucleotide sequence, the label is normally bound to an sbp member complementary to an sbp member that is bound to or part of a nucleotide sequence. Other components of the signal producing system may be included in a developer solution and can include substrates, enhancers, activators, chemiluminescent compounds, cofactors, inhibitors, scavengers, metal ions, specific binding substances required for binding of signal generating substances, and the like. Other components of the signal producing system may be coenzymes, substances that react with enzymic products, other enzymes and catalysts, and the like. The signal producing system provides a signal detectable by external means, by use of electromagnetic radiation, desirably by visual examination. The signal-producing system is described more fully in U.S. Patent No. 5,508,178 (Rose, *et al.*), the relevant disclosure of which is incorporated herein by reference.

Ancillary materials – Various ancillary materials will frequently be employed in the methods and assays carried out in accordance with the present invention. For example, buffers will normally be present in the assay medium, as well as stabilizers for the assay medium and the assay components. Frequently, in addition to these additives, proteins may be included, such as albumins, organic solvents such as formamide, quaternary ammonium salts, polycations such as dextran sulfate, surfactants, particularly non-ionic surfactants, binding enhancers, e.g., polyalkylene glycols, or the like.

As mentioned above, one aspect of the present invention is a method for amplifying nucleic acids wherein a modified oligonucleotide having a portion that is complementary to the nucleic acid is employed having at its 3'-end a group that can be catalytically removed by an enzyme. The group is not capable of being extended upon by the

polymerase used in an amplification and must not be removed as rapidly as a natural nucleotide in a corresponding oligonucleotide.

In the method an oligonucleotide primer is controllably generated and selectively extended along a target polynucleotide sequence in a mixture of polynucleotides. The mixture is provided in combination with a modified oligonucleotide having a 3'-end that is not extendable along any polynucleotide sequence to which it might bind. Controlled generation of an oligonucleotide primer is achieved by controlling the degradation of the 3'-end of the modified oligonucleotide to produce degraded modified oligonucleotide or oligonucleotide primer. In this way, small amounts of oligonucleotide primer are generated *in situ* and the generated oligonucleotide primer selectively binds to, and is extended along, the target polynucleotide sequence. Again, this is achieved by controlling the degradation of the 3'-end of the modified oligonucleotide. Since small amounts of the oligonucleotide primer are controllably generated and because of the binding specificity of the generated oligonucleotide primer for the target polynucleotide sequence, binding of the oligonucleotide primer to irrelevant polynucleotides is substantially reduced. Accordingly, extension of oligonucleotide primer along any polynucleotides in the reaction mixture other than the target polynucleotide sequence is avoided. Controlled degradation of the modified oligonucleotide provides for production of oligonucleotide primer that is extendable. Controlled production of the oligonucleotide primer provides for hybridization of such primer selectively to a target polynucleotide sequence and not to irrelevant polynucleotides because the production of such extendable primer occurs slowly and, therefore, its concentration at any point in time is low enough to permit selective hybridization to target polynucleotide sequence. Control of degradation may also be accomplished by selection of the type of modification in the



modified oligonucleotide and the amount of the agent that removes the modification such as  $\text{exo}^+$  polymerase, and esterase or light.

One embodiment of the present invention is depicted in Fig. 1. In this embodiment an amplification of a target polynucleotide sequence (TPS) by PCR amplification is chosen by way of example and not  
5 limitation. TPS is combined in a suitable buffered aqueous medium with modified oligonucleotide MO1 and oligonucleotide primer OP2, which are capable of hybridizing to one or the other strands of the double stranded TPS. MO1 contains portion MN1 that cannot hybridize with  
10 TPS because MN1 contains at least one modified nucleotide that is capable of being slowly degraded by a 3' to 5' exonuclease. Accordingly, MO1, when hybridized to TPS, cannot be extended along TPS, nor along any irrelevant DNA to which it might hybridize. Also included in the medium are nucleoside triphosphates (NTP's) and a  
15 nucleotide polymerase having 3' to 5' exonuclease activity (NP-exo). The temperature of the medium is relatively low, for example, being about 25°C to 45°C. The ability of the 3' to 5' exonuclease to degrade portion MN1 of MO1 to give OP1 is increased as the temperature of the reaction medium is increased. Accordingly, as the temperature is raised,  
20 duplexes are denatured and MO1 is degraded to remove the modified nucleotides MN1F. As the temperature is lowered to about 70°C to 80°C during the next cycle and in the presence of the nucleoside triphosphates and nucleotide polymerase, OP1 is extended along the strand of TPS to which it selectively hybridizes to produce extended OP1  
25 (EOP1). At the elevated temperature binding of nucleotide sequences to one another is more selective so that OP1, which is present in a relatively low concentration, selectively binds to TPS and the amount that may be bound to irrelevant DNA is very substantially reduced. As a result background products are greatly decreased. OP2 is also  
30 extended along the strand of TPS to which it is hybridized to produce

extended OP2 (EOP2). Thermal cycling results in the continued controlled formation of OP1 from MO1, which leads to the production of multiple copies of TPS. Control of the temperature thus results in preferential extension of OP1 along TPS due to the controlled  
5 degradation of the modified portion of MO1.

To enhance the effect achieved in PCR through application of the present invention, MO2 containing modified portion MN2 is used in place of the unmodified OP2. Referring to Fig. 2, TPS is combined in a suitable buffered aqueous medium with two different oligonucleotide  
10 primers, modified oligonucleotide primer MO1 and modified oligonucleotide primer MO2, which are respectively capable of hybridizing to one of the strands of the double stranded TPS. As with MO1 above, MO2 contains portion MN2 that cannot hybridize with TPS because MN2 contains at least one modified nucleotide that is capable  
15 of being slowly degraded by a 3' to 5' exonuclease. Accordingly, MO2 cannot be extended along TPS, nor along any irrelevant DNA to which it might hybridize. Also included in the medium are nucleoside triphosphates (NTP's) and a nucleotide polymerase having 3' to 5' exonuclease activity (NP-exo). The temperature of the medium is  
20 relatively low, for example, being about 20°C to 45°C. The ability of the 3' to 5' exonuclease to degrade portion MN1 of MO1 and portion MN2 of MO2 is increased as the temperature of the reaction medium is increased. Accordingly, as the temperature is raised, duplexes are denatured, MO1 is degraded to remove the modified nucleotides MN1F  
25 and MO2 is degraded to remove modified nucleotides MN2F. As the temperature is lowered to about 70°C to 80°C during the next cycle and in the presence of the nucleoside triphosphates and nucleotide polymerase, OP1 is extended along the strand of TPS to which it is hybridized to produce extended OP1 (EOP1) and OP2 is also  
30 extended along the strand of TPS to which it is hybridized to produce

extended OP2 (EOP2). As above in the embodiment of Fig 1, continued thermal cycling thus leads to the production of multiple copies of TPS. Control of the temperature thus results in preferential extension of OP1 and OP2 along TPS due to the controlled degradation of the modified  
5 portion of MO1 and MO2.

In applying the present invention to PCR amplification of nucleic acids, generally the reaction medium is cycled between two to three temperatures. The general principle in the present invention is that hybridization of the oligonucleotide primer to the target polynucleotide  
10 sequence take place only at elevated temperature where binding is relatively selective. Thus, binding of the oligonucleotide primer to irrelevant polynucleotides is minimized. Normally, in conducting the methods the medium is cycled between two or three temperatures. The temperatures for the methods generally range from about 10 to 105°C, more usually from  
15 about 40 to 99°C, preferably 50 to 98°C. The exact temperatures can be varied depending on the salt concentration, pH, solvents used, length of and composition of the target polynucleotide sequence and the primer. Relatively low temperatures of from about 30 to 65°C can be employed for the extension steps, while denaturation and hybridization can be carried  
20 out at a temperature of from about 50 to 105°C. As mentioned above the reaction medium is initially at about 20°C to 45°C, preferably, about 25°C to 35°C. Relatively low temperatures of from about 50°C to 80°C, preferably, 50°C to 60°C, are employed for the hybridization or annealing steps, while denaturation is carried out at a temperature of from about  
25 80°C to 100°C, preferably, 90°C to 95°C, and extension is carried out at a temperature of from about 70°C to 80°C, usually about 72°C to 74°C.

The amplification is conducted for a time sufficient to achieve a desired number of copies. Generally, the time period for conducting the method is from about 10 seconds to 10 minutes per cycle and any  
30 number of cycles can be used from 1 to as high as 60 or more, usually

10 to 50, frequently, 20 to 45. As a matter of convenience it is usually desirable to minimize the time period and the number of cycles. In general, the time period for a given degree of amplification can be minimized, for example, by selecting concentrations of nucleoside triphosphates sufficient to saturate the polynucleotide polymerase, by  
5 increasing the concentrations of polynucleotide polymerase and polynucleotide primer, and by using a reaction container that provides for rapid thermal equilibration. Generally, the time period for conducting the amplification in the method of the invention is from about 5 to 200  
10 minutes. As a matter of convenience, it will usually be desirable to minimize the time period.

In carrying out the methods in accordance with the present invention, including amplification, an aqueous medium is employed. Other polar cosolvents may also be employed, usually oxygenated  
15 organic solvents of from 1-6, more usually from 1-4, carbon atoms, including alcohols, ethers and the like. Usually these cosolvents, if used, are present in less than about 70 weight percent, more usually in less than about 30 weight percent.

The pH for the medium is usually in the range of about 4.5 to 9.5, more usually in the range of about 5.5 - 8.5, and preferably in the range  
20 of about 6 - 8. The pH and temperature are chosen and varied, as the case may be, so as to cause, either simultaneously or sequentially, dissociation of any internally hybridized sequences, hybridization of oligonucleotide primer with the target polynucleotide sequence,  
25 degradation of the 3'-end of the oligonucleotide primer hybridized to the target polynucleotide sequence, extension of the primer(s), and dissociation of the extended primer(s). In some instances, a compromise is made in optimizing the speed, efficiency, and specificity of these steps depending on whether it is desired to perform the above  
30 steps sequentially or simultaneously. Various buffers may be used to

achieve the desired pH and maintain the pH during the determination. Illustrative buffers include borate, phosphate, carbonate, Tris, barbital and the like. The particular buffer employed is not critical to this invention but in individual methods one buffer may be preferred over  
5 another.

The concentration of the enzyme having 3' to 5' exonuclease activity is sufficient to realize the requisite level of degradation of the oligonucleotide primer containing the modified nucleotide(s). The concentration is usually about 0.1 to 10 units per one hundred microliter  
10 reaction volume, preferably, 1 to 5 units per one hundred microliter reaction volume. Where such enzyme also functions as a polymerase, the concentration of this polymerase will be chosen to be sufficient to accomplish chain extension. The concentration of the polymerase is usually determined empirically. Preferably, a concentration is used that  
15 is sufficient such that further increase in the concentration does not decrease the time for the amplification by over 5-fold, preferably 2-fold. The primary limiting factor generally is the cost of the reagent. Where the exonuclease activity of the enzyme is great enough to result in premature degradation of the modified oligonucleotide primer at low  
20 temperature, the corresponding enzyme lacking exonuclease activity may be included in the reaction medium to provide, in effect, a dilution of the exonuclease activity of the overall concentration of enzyme. For example, if the enzyme used is Pfu polymerase, Pfu exo<sup>-</sup> may be included in the reaction medium in place of a portion of the Pfu  
25 polymerase. The amount of enzyme lacking exonuclease activity used is generally determined empirically as the amount that achieves an insubstantial level of degradation of the modified oligonucleotide primer at low temperature.

The amount of the target polynucleotide sequence that is to be  
30 copied can be as low as one or two molecules in a sample but generally

may vary from about  $10^2$  to  $10^{10}$ , more usually from about  $10^3$  to  $10^8$  molecules in a sample, preferably at least  $10^{-21}$ M in the sample and may be  $10^{-10}$  to  $10^{-19}$ M, more usually  $10^{-14}$  to  $10^{-19}$ M.

5           The amount of the modified oligonucleotide is governed by the amount of oligonucleotide primer needed for the particular amplification or other reaction to which the present invention is applied. The amount of oligonucleotide primer(s) will be at least as great as the number of copies desired and will usually be  $1 \times 10^{-10}$  to  $1 \times 10^{-6}$  moles per sample,  
10       where the sample is 1-1,000  $\mu$ L. Usually, the primer(s) are present in at least 0.1  $\mu$ M, preferably 0.5  $\mu$ M, and more preferably at least about 1  $\mu$ M. Preferably, the concentration of the oligonucleotide primer(s) is substantially in excess over, preferably at least  $1 \times 10^{14}$  times greater than, the concentration of the target polynucleotide sequence.

15           The concentration of the deoxynucleoside triphosphates in the medium can vary widely; preferably, these reagents are present in an excess amount. The deoxynucleoside triphosphates are usually present at  $10^{-6}$  to  $10^{-2}$ M, preferably  $10^{-5}$  to  $10^{-3}$ M.

          The order of combining of the various reagents to form the  
20       combination may vary. Generally, the target polynucleotide sequence is obtained from a sample containing such sequence or a polynucleotide analyte that has been treated to obtain such sequence. Generally, the target polynucleotide sequence is combined with a pre-prepared combination of deoxynucleoside triphosphates and polymerase. The  
25       oligonucleotide primer(s) may be included in the prepared combination or may be added subsequently. However, simultaneous addition of all of the above, as well as other step-wise or sequential orders of addition, may be employed.

          The concentration and order of addition of reagents and  
30       conditions for the method are governed generally by the desire to

maximize the number of copies of the extended primer(s) and the rate at which such copies are formed and the fidelity of replication. Generally, it is desirable to increase the number of copies of the extended primer by at least a factor of  $10^2$ , preferably a factor of  $10^4$ , more preferably  $10^6$  or  
5 more.

In carrying out the method of the invention as applied to the detection of a polynucleotide analyte, the considerations as to media, pH, temperature, and times can be as described above.

While the concentrations of the various reagents are generally  
10 determined by the concentration range of interest of the polynucleotide analyte, the final concentration of many of the reagents is normally determined empirically to optimize the sensitivity of the assay over the range of interest. The concentration of the other reagents in an assay generally is determined following the same principles as set forth above.  
15 The primary consideration is that a sufficient number of copies of extended primer(s) be produced in relation to the polynucleotide analyte sequence so that such copies can be readily detected and provide an accurate determination of the target polynucleotide sequence.

The copies of extended primer(s) can be detected in numerous  
20 ways. For example, in the present method, molecules of the oligonucleotide primer can be labeled with a reporter molecule such as a ligand, a small organic molecule, a polynucleotide sequence, a protein, support, a member of an operator-repressor pair, intercalation dye and the like. Any standard method for specifically detecting nucleic acid.  
25 sequences can be used. Gel electrophoresis for detecting chain extension may be employed.

One method for detecting nucleic acids is to employ nucleic acid probes. One method utilizing probes is described in U.S. Patent No. 4,868,104, the disclosure of which is incorporated herein by reference.

Other assay formats and detection formats are disclosed in U.S. Patent No. 5,508,178, U.S. Patent No. 5,439,998 and U.S. Patent Application Serial No. 07/776,538 filed October 11, 1991, which have been incorporated herein by reference.

5           Examples of particular labels or reporter molecules and their detection can be found in U.S. Patent No. 5,439,998, the relevant disclosure of which is incorporated herein by reference.

          Detection of the signal will depend upon the nature of the signal producing system utilized. If the label or reporter group is an enzyme,  
10   additional members of the signal producing system would include enzyme substrates and so forth. The product of the enzyme reaction is preferably a luminescent product, or a fluorescent or non-fluorescent dye, any of which can be detected spectrophotometrically, or a product that can be detected by other spectrometric or electrometric means. If  
15   the label is a fluorescent molecule the medium can be irradiated and the fluorescence determined. Where the label is a radioactive group, the medium can be counted to determine the radioactive count.

          The present method has application where the target polynucleotide sequence is DNA or RNA.

20           In one aspect of the invention one or more of the reagents, such as, for example, a modified oligonucleotide and/or an oligonucleotide primer, is labeled with a label (reporter molecule). The reporter molecule can be, for example, a detectable group or a binder such as biotin or a nucleotide sequence other than the sequence that hybridizes with the  
25   target sequences. The extended primer(s) can be detected by means of a reporter molecule covalently bonded to a probe. The probe has a nucleotide sequence that is homologous or complementary to a portion of the target nucleotide sequence other than those sequences to which the primers bind.



The present invention also has application to amplification using a single oligonucleotide primer as described in U.S. Patent No. 5,508,178 and U.S. Patent Application Serial No. 08/140,349 filed October 20, 1993. This embodiment is depicted in Fig. 3. A polynucleotide

5 comprises a target polynucleotide sequence TPS and includes a first sequence of nucleotides designated primer binding site PS1 and a second sequence of nucleotides designated S2. The polynucleotide is combined in a suitable buffered aqueous medium with a single modified oligonucleotide MO3, which contains a sequence of nucleotides OP3

10 that is capable of hybridizing to PS1. MO3 contains portion MN3 that cannot hybridize with TPS because MN3 contains at least one modified nucleotide that is capable of being slowly degraded by a 3' to 5' exonuclease. Accordingly, MO3 cannot be extended along TPS, nor along any irrelevant DNA to which it might hybridize. Also included in

15 the medium are nucleoside triphosphates (NTP's), a strand switch blocker (SSB) oligonucleotide (in accordance with the method of U.S. Patent Application Serial No. 08/140,349 filed October 20, 1993, referred to above) and a nucleotide polymerase having 3' to 5' exonuclease activity (NP-exo). The temperature of the medium is relatively low, for

20 example, being about 25 to 45°C. The ability of the 3' to 5' exonuclease to degrade portion MN3 of MO3 to give OP3 is increased as the temperature of the reaction medium is increased. Accordingly, as the temperature is raised, duplexes are denatured and MO3 is degraded to remove the modified nucleotides MN3F. As the temperature is lowered

25 to about 70°C to 80°C during the next cycle and in the presence of the nucleoside triphosphates, the strand switch blocker SSB and nucleotide polymerase, OP3 is extended along TPS to the point at which OP3 encounters SSB. At this point OP3 extends along that portion of SSB that is not hybridized to TPS. As a result extended OP3 (EOP3) is

30 formed that includes sequence OP3 and sequence PS1, which is

complementary to OP3 as well as TPS. EOP3 is then amplified during the thermal cycling by the single primer OP3, produced in a controlled manner by the degradation of MO3 in accordance with the present invention. Molecules of EOP3 serve as templates for primer OP3.

- 5 Continued thermal cycling leads to the production of multiple copies of TPS. Control of the temperature thus results in preferential extension of OP3 along TPS due to the controlled degradation of the modified portion of MO3.

The present invention also has application to a method for detecting  
10 differences in related nucleic acid sequences. The method involves chain extension of oligonucleotide primers and is described in U.S. Patent Application Serial No. 60/009,289, the disclosure of which was incorporated herein by reference above. Briefly, a combination of reagents is formed in the same reaction medium. The combination comprises (i) a  
15 sample containing a target nucleic acid sequence suspected of having a mutation, (ii) a reference nucleic acid sequence, which may be added separately if it is not known to be present in the sample and which corresponds to the target nucleic acid lacking the mutation, which may be the wild type nucleic acid, (iii) a nucleotide polymerase, (iv) nucleoside  
20 triphosphates, and (v) three oligonucleotide primers where two sets of one of the primers each may be labeled with different labels. The medium is then subjected to multiple temperature cycles of heating and cooling to simultaneously achieve all of the amplification and chain extension reactions. Preferably, in this embodiment, each cycle includes heating the  
25 medium at 90°C to 100°C for 10 seconds to 3 minutes, cooling the medium to 60°C to 70°C for a period of 10 seconds to 3 minutes, and heating the medium at 70°C to 75°C for a period of 10 seconds to 3 minutes although different temperatures may be required depending on the lengths of the primer sequences. Following the above temperature cycling the medium is  
30 subjected to heating for a period of time sufficient to denature double

stranded molecules, preferably, at 90°C to 99°C for 10 seconds to 2 minutes, and cooled to 40°C to 80°C, preferably 60°C to 70°C, and held at this temperature for at least one minute, preferably for 20 minutes to 2 hours.

5           Following cooling of the medium (see Fig. 4), all possible partial and complete duplexes are formed that can form from 1) single strands that have any combination of reference or mutant sequences and 5'-ends A2 and B2, and 2) single strands having any combination of reference or mutant sequences and 5'-ends A1 or B1 wherein the strands may further  
10 be labeled with either L1 or L2 when L1 and L2 are different. Among the partial duplexes that are formed are the tailed partial duplexes A' and B', which can bind to each other to form complex C, which does not dissociate into duplexes D and E when a mutation is present. A determination of the presence of such a complex is then made to establish the presence of a  
15 mutation in the target nucleic acid sequence.

Referring to Fig. 4, the above reactions that occur simultaneously are described in a step-wise manner. In this embodiment with application of the present invention, three modified oligonucleotides are employed and are designated MO4, MO5 and MO6, respectively. In the embodiment  
20 shown in Fig. 4, by way of illustration and not limitation, two sets of modified oligonucleotide MO5 are employed wherein one set is labeled with L1 and the other set is labeled with L2. A tailed target partial duplex A' is produced from target nucleic acid duplex A having mutation M and tailed reference partial duplex B' is produced from reference nucleic acid  
25 duplex B.

Referring to Fig. 4, target nucleic acid A and reference nucleic acid B are combined in a suitable buffered aqueous medium with the modified oligonucleotides, MO4, MO5-L1 and MO5-L2, and MO6. In accordance with the present invention MO4 contains portion MN4 that  
30 does not hybridize to its respective strand of A and MO5-L1 contains

portion MN5 that does not hybridize to its respective strand of A. Likewise, MO6 contains portion MN6 that does not hybridize to its respective strand of B and MO5-L2 contains portion MN5 that does not hybridize to its respective strand of B. Each of the portions MN4, MN5 and MN6 contain at least one modified nucleotide that is capable of being slowly degraded by a 3' to 5' exonuclease. Accordingly, MO4, MO5 and MO6 cannot be extended along the respective strands of A or B, nor along any irrelevant DNA to which they might hybridize.

Also included in the medium are nucleoside triphosphates (NTP's) and a nucleotide polymerase having 3' to 5' exonuclease activity (NP-EXO). The temperature of the medium is relatively low, for example, being about 25 to 45°C. The ability of the 3' to 5' exonuclease to degrade portion MN4 of MO4, MN5 of MO5 and MN6 of MO6 is increased as the temperature of the reaction medium is increased. Accordingly, as the temperature is raised, duplexes are denatured, MO4 is degraded to remove the modified nucleotides MN4F, MO5 is degraded to remove the modified nucleotides MN5F and MO6 is degraded to remove modified nucleotides MN6F. As the temperature is lowered to about 70°C to 80°C during the next cycle and in the presence of the nucleoside triphosphates and nucleotide polymerase, OP4, OP5 and OP6 are extended along the respective strands of A or B to which each is respectively hybridized. At the elevated temperature binding of nucleotide sequences to one another is more selective so that OP4, OP5 and OP6, which are present in a relatively low concentration, selectively binds to their respective strands of A and B so that the level at which OP4, OP5 and OP6 may be bound to irrelevant DNA is substantially reduced. Thus, consistent with the present invention, background products are greatly decreased.

As depicted in Fig. 4, A is amplified by the polymerase chain reaction using primers OP4 and OP5 to produce an amplicon AA. Primer OP5 contains a label L1 and primer OP4 is comprised of a 3'-end portion

Pa that can hybridize with the target sequence and 5'-end portion B1 that cannot hybridize with the target sequence. The amplification is carried out in the presence of a nucleotide polymerase and nucleoside triphosphates using temperature cycling. Amplicon AA has two strands, a labeled strand  
5 derived from primer OP5 and an unlabeled strand derived from primer OP4. The unlabeled strand has a 5'-end portion B1 of primer OP4 and the labeled strand has a corresponding 3'-end portion A2, which is the complement of B1. Referring again to Fig. 4, chain extension of primer  
10 OP6 along the labeled strand of amplicon AA occurs to produce tailed target partial duplex A'. Primer OP6 is comprised of a 3'-end portion Pa, which is identical to Pa of primer OP4 and which binds to the labeled strand of AA. OP6 has 5'-end portion A1 that is not complementary to amplicon AA. In the embodiment of Fig. 4, the important strand is the complementary strand of the labeled strand and not its copy. The  
15 complementary unlabeled strand of tailed target partial duplex A' has a 5'-end portion A1, which is not complementary to the 3'-end portion A2 of the labeled strand of A'.

Again referring to Fig. 4, reference nucleic acid sequence B is comprised of a sequence identical to A except for mutation M. Primer OP5  
20 contains label L2 that is different than L1. Amplicon BB has two strands, a labeled strand derived from the extension of primer OP5-L2 and an unlabeled strand derived from the extension of primer OP6. The unlabeled strand has end portion A1 of primer OP6 and the labeled strand has corresponding end portion B2, which is the complement of A1.

25 Chain extension of primer OP4 along the labeled strand of amplicon BB produces tailed reference partial duplex B'. As mentioned above, primer OP4 is comprised of portion Pa, which binds to the labeled strand of BB and portion B1 that does not bind to amplicon BB. The extension product of primer OP4 has a 5'-end portion B1, which is not  
30 complementary to end portion B2 of the labeled strand of B'. As can be

seen, A' and B' are related in that each of their labeled strands is complementary, except for mutation M, to the unlabeled strand of the other.

5 The strands of partial duplexes A' and B' bind and undergo branch migration under the reaction conditions, for example, a temperature of 30°C to 75°C, preferably 60°C to 70°C, for at least one minute, preferably, 20 to 40 minutes, wherein complex C is formed. Oligonucleotide tail A1 of A' hybridizes to corresponding oligonucleotide tail B2 of B' and, similarly, oligonucleotide tail A2 of A' is hybridizes to oligonucleotide tail B1 of B'.  
10 Branch migration within complex C continues under the above temperature conditions with separation of the complex into duplexes D and E unless a mutation M is present, whereupon branch migration and strand dissociation is inhibited. Complex C is then detected, the presence of which is directly related to the presence of mutation M.

15 In the embodiment depicted in Fig. 4, labels L1 and L2 are incorporated into the partial duplexes that comprise complex C and provide a means for detection of complex C. This is by way of illustration and not limitation and other convenient methods for detecting complex C may be employed, such as the use of a receptor for the complex. In this approach  
20 there is required only one label, L1 or L2, which comprises an sbp member or a reporter molecule. A receptor for the sbp member and a receptor that can bind to complex C by virtue of a feature other than L1 or L2 can both bind to complex C and provide a means for detection.

The conditions for carrying out the detection of differences in nucleic  
25 acids wherein the present invention is utilized are similar to those for the amplification described above. In general, the medium is heated to a temperature of 90°C to 100°C for a period of 5 to 500 seconds and then cooled to 20°C to 80°C for a period of 5 to 2000 seconds followed by heating to 40°C to 80°C for a period of 5 to 2000 seconds. Preferably, the  
30 medium is subjected to heating at 90°C to 100°C for a period of 10

seconds to 3 minutes, cooling to 50°C to 65°C for a period of 10 seconds to 2 minutes and heating to 70°C to 80°C for a period of 30 seconds to 5 minutes.

As a matter of convenience, predetermined amounts of reagents employed in the present invention can be provided in a kit in packaged combination. A kit can comprise in packaged combination an oligonucleotide primer comprising at least one nucleotide analog at the 3'-end thereof, nucleotide triphosphates and a 3' to 5' exonuclease. The nucleotide analog of the oligonucleotide primer is substantially non-hybridizable to any normal nucleotide in a polynucleotide sequence. In one embodiment the nucleotide analog has a 3'-hydroxyl group. In another embodiment the nucleotide analog is a natural nucleotide having a chemical modification that is removable. Preferably, the nucleotide analog is removable by an enzyme having 3' to 5' exonuclease activity at a rate that is slower than for a natural nucleotide. In the event that a nucleotide polymerase is included in the kit and the nucleotide polymerase does not have 3' to 5' exonuclease activity, then the kit further comprises an enzyme having 3' to 5' exonuclease activity.

A kit for amplification of a target polynucleotide sequence comprises the above items and for conducting PCR would include in addition a second polynucleotide primer, where the primers are related in that a product of the extension of one along said target sequence serves as a template for the extension of the other.

In assaying for a polynucleotide analyte in a sample, a kit useful in the present method can comprise, in packaged combination with other reagents mentioned above, reagents for forming a target polynucleotide sequence from a polynucleotide analyte. Furthermore, the oligonucleotide primer can be labeled or can be provided with groups to render the sequence labeled or bound to a support. The kit can further include a labeled polynucleotide probe capable of binding to an amplified

target polynucleotide sequence. The kit can further include members of a signal producing system and also various buffered media, some of which may contain one or more of the above reagents.

- 5           The relative amounts of the various reagents in the kits can be varied widely to provide for concentrations of the reagents which substantially optimize the reactions that need to occur during the present method and to further substantially optimize the sensitivity of the assay. Under appropriate circumstances one or more of the reagents in the kit
- 10       can be provided as a dry powder, usually lyophilized, including excipients, which on dissolution will provide for a reagent solution having the appropriate concentrations for performing a method or assay in accordance with the present invention. Each reagent can be packaged in separate containers or some reagents can be combined in one
- 15       container where cross-reactivity and shelf life permit. The kit can further include a written description of a method in accordance with the present invention as described above.

#### EXAMPLES

- 20           The invention is demonstrated further by the following illustrative examples. Temperatures are in degrees centigrade (°C) and parts and percentages are by weight, unless otherwise indicated.

          The following definitions and abbreviations are used herein:

- Tris - Tris(hydroxymethyl)aminomethane-HCl (a 10X solution) from
- 25       BioWhittaker, Walkersville, MD.
- HPLC - high performance liquid chromatography.
- DTT - dithiothreitol
- BSA - bovine serum albumin from Gibco BRL, Gaithersburg MD
- bp - base pairs
- 30       wt (+) - wild type allele



mut (-) - mutant allele

Target sample - DNA sample to be tested for the presence of a mutation;

sec - seconds

5 hr - hours

min - minutes

Buffer A - 10mM Tris-HCl (pH8.3), 50mM KCl, 4mM MgCl<sub>2</sub>,  
200 µg/ml BSA

10 Buffer C - 0.1M Tris, 0.3M NaCl, 25 mM EDTA, 0.1% BSA, 0.1%  
dextran T-500, a 1:320 dilution of mouse IgG (HBR-1 from Scantibodies  
Laboratory Inc., Los Angeles, CA), 0.05% Kathon (Rohm and Haas,  
Philadelphia, PA), and 0.01% gentamycin sulfate.

RLU - relative light units

nt - nucleotides

15 MAD - maleimidylaminodextran

Ab - antibody

Sav - streptavidin

MOPS - 3-(N-morpholino)propane sulfonic acid

hr - hour

20 sulfo-SMCC - sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-  
1-carboxylate

NHS - N-hydroxysuccinimide

EDAC - 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide  
hydrochloride

25 DMSO - dimethylsulfoxide

MES - morpholinoethanesulfonate

rpm - rotations per min

EDTA - ethylenediaminetetraacetic acid

SATA - N-succinimidyl S-acetylthioacetate

BSA - bovine serum albumin from Sigma Chemical Company, St.  
Louis MO

eq - equivalents

bp - base pairs

5  $A_{280}$  - absorbance at wavelength 280 nanometers

DexAl - dextran aldehyde

DPP - 4,7-diphenylphenanthroline

Eu(TTA)<sub>3</sub> - europium tri-3-(2-thienoyl)-1,1,1-trifluoroacetate

L or l - liter

10 exo VII - exonuclease VII from E.coli (from Amersham Life Science)  
(USB).

DMF - dimethyl formamide

THF - tetrahydrofuran

MS - mass spectroscopy

15 NMR - nuclear magnetic resonance spectroscopy

TMSCl - tetramethylsilylchloride

ELISA - enzyme linked immunosorbent assay as described in  
"Enzyme-Immunoassay," Edward T. Maggio, CRC Press, Inc., Boca  
Raton, Florida (1980)

20 Monoclonal antibodies were produced by standard hybrid cell  
technology. Briefly, the appropriated immunogen was injected into a host,  
usually a mouse or other suitable animal, and after a suitable period of  
time the spleen cells from the host were obtained. Alternatively,  
unsensitized cells from the host were isolated and directly sensitized with  
25 the immunogen in vitro. Hybrid cells were formed by fusing the above cells  
with an appropriate myeloma cell line and culturing the fused cells. The  
antibodies produced by the cultured hybrid cells were screened for their  
binding affinity to the particular antigen, dig-BSA conjugate. A number of  
screening techniques were employed such as, for example, ELISA  
30 screens. Selected fusions were then recloned.

Beads:

Acc-AbDig - Acceptor beads coupled (MAD) to the anti-Dig antibody

(with 377 antibody molecules per bead) were prepared as follows:

- 5        Hydroxypropylaminodextran (1NH<sub>2</sub>/ 7 glucose) was prepared by dissolving Dextran T-500 (Pharmacia, Uppsala, Sweden) (50g) in 150 mL of H<sub>2</sub>O in a 3-neck round-bottom flask equipped with mechanical stirrer and dropping funnel. To the above solution was added 18.8g of Zn (BF<sub>4</sub>)<sub>2</sub> and the temperature was brought to 87°C with a hot water bath.
- 10       Epichlorohydrin (350mL) was added dropwise with stirring over about 30 min while the temperature was maintained at 87-88°C. The mixture was stirred for 4 hr while the temperature was maintained between 80°C and 95°C, then the mixture was cooled to room temperature. Chlorodextran product was precipitated by pouring slowly into 3L of methanol with
- 15       vigorous stirring, recovered by filtration and dried overnight in a vacuum oven.

- The chlorodextran product was dissolved in 200mL of water and added to 2L of concentrated aqueous ammonia (36%). This solution was stirred for 4 days at room temperature, then concentrated to about 190mL
- 20       on a rotary evaporator. The concentrate was divided into two equal batches, and each batch was precipitated by pouring slowly into 2L of rapidly stirring methanol. The final product was recovered by filtration and dried under vacuum.

- Hydroxypropylaminodextran (1NH<sub>2</sub>/ 7 glucose), prepared above,
- 25       was dissolved in 50mM MOPS, pH 7.2, at 12.5 mg/mL. The solution was stirred for 8 hr at room temperature, stored under refrigeration and centrifuged for 45 min at 15,000 rpm in a Sorvall RC-5B centrifuge immediately before use to remove a trace of solid material. To 10mL of this solution was added 23.1mg of Sulfo-SMCC in 1mL of water. This

mixture was incubated for 1 hr at room temperature and used without further purification.

C-28 thioxene was prepared as follows:

To a solution of 4-bromoaniline (30g, 174mmol) in dry DMF (200mL) was  
5 added 1-bromotetradecane (89.3mL, 366mmol) and N,N-  
diisopropylethylamine (62.2mL, 357mmol). The reaction solution was  
heated at 90°C for 16 hr under argon before being cooled to room  
temperature. To this reaction solution was again added 1-  
bromotetradecane (45mL, 184mmol) and N,N-diisopropylethylamine  
10 (31mL, 178mmol) and the reaction mixture was heated at 90°C for another  
15 hr. After cooling, the reaction solution was concentrated in vacuo and  
the residue was diluted with CH<sub>2</sub>Cl<sub>2</sub> (400mL). The CH<sub>2</sub>Cl<sub>2</sub> solution was  
washed with 1N aqueous NaOH (2x), H<sub>2</sub>O, and brine, was dried over  
Na<sub>2</sub>SO<sub>4</sub> and was concentrated in vacuo to yield a dark brown oil (about  
15 110g). Preparative column chromatography on silica gel by a Waters 500  
Prep LC system eluting with hexane afforded a yellow oil that contained  
mainly the product (4-bromo-N,N-di-(C<sub>14</sub>H<sub>29</sub>)-aniline) along with a minor  
component 1-bromotetradecane. The latter compound was removed from  
the mixture by vacuum distillation (bp 105-110°C, 0.6mm) to leave 50.2g  
20 (51%) of the product as a brown oil. To a mixture of magnesium turnings  
(9.60g, 395mmol) in dry THF (30mL) under argon was added dropwise a  
solution of the above substituted aniline product (44.7g, 79mmol) in THF  
(250mL). A few crystals of iodine were added to initiate the formation of  
the Grignard reagent. When the reaction mixture became warm and  
25 began to reflux, the addition rate was regulated to maintain a gentle reflux.  
After addition was complete, the mixture was heated at reflux for an  
additional hour. The cooled supernatant solution was transferred via  
cannula to an addition funnel and added dropwise (over 2.5 hr) to a  
solution of phenylglyoxal (11.7g, 87mmol) in THF (300mL) at -30°C under  
30 argon. The reaction mixture was gradually warmed to 0°C over 1 hr and

stirred for another 30 min. The resulting mixture was poured into a mixture of ice water (800mL) and ethyl acetate (250mL). The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3x). The combined organic phases were washed with H<sub>2</sub>O (2x), brine and was  
5 dried over MgSO<sub>4</sub>. Evaporation of the solvent gave 48.8g of the crude product as a dark green oily liquid. Flash column chromatography of this liquid (gradient elution with hexane, 1.5:98.5, 3:97, 5:95 ethyl acetate:hexane) afforded 24.7g (50%) of the benzoin product (MS (C<sub>42</sub>H<sub>69</sub>NO<sub>2</sub>): [M-H]<sup>+</sup> 618.6, <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) was consistent with  
10 the expected benzoin product. To a solution of the benzoin product from above (24.7g, 40mmol) in dry toluene (500mL) was added sequentially 2-mercaptoethanol (25g, 320mmol) and TMSCl (100mL, 788mmol). The reaction solution was heated at reflux for 23 hr under argon before being cooled to room temperature. To this was added additional TMSCl (50mL,  
15 394mmol) ;and the reaction solution was heated at reflux for another 3 hr. The resulting solution was cooled, was made basic with cold 2.5N aqueous NaOH and was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3x). The combined organic layers were washed with saturated aqueous NaHCO<sub>3</sub> (2x) and brine, was dried over Na<sub>2</sub>SO<sub>4</sub> and was concentrated in vacuo to give a brown oily liquid.  
20 Preparative column chromatography on silica gel by using a Waters 500 Prep LC system (gradient elution with hexane, 1:99, 2:98 ethyl acetate:hexane) provided 15.5g (60%) of the C-28 thioxene as an orange-yellow oil (MS (C<sub>44</sub>H<sub>71</sub>NOS): [M-H]<sup>+</sup> 661.6, <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) was consistent with the expected C-28 thioxene product 2-(4-(N,N-di-(C<sub>14</sub>H<sub>29</sub>)-anilino)-3-phenyl thioxene.  
25

Carboxyl acceptor beads were prepared as follows:

The starting beads were carboxylate modified latex purchased from Seradyn Particle Technology, Indianapolis, IN. The beads contained Eu(TTA)<sub>3</sub>DPP prepared as follows: DPP/Eu(TTA)<sub>3</sub> was prepared by  
30 combining 8.69g of Eu(TTA)<sub>3</sub> · 3H<sub>2</sub>O (10 mmoles, Kodak Chemical

Company, Rochester NY) and 1.8g of 1,10-phenanthroline (10 mmoles, Aldrich) in 50ml of dry toluene and heating to 95°C in an oil bath for one hour. Toluene was removed under reduced pressure. The ash colored solid was crystallized from 10ml of toluene to yield 10 grams of

- 5 DPP/Eu(TTA)<sub>3</sub>. Absorption spectrum: 270 nm (20,000), 340 nm (60,000) (Toluene) 1.R(KBr):  $\text{Cm}^{-1}$ : 3440(s), 1600(s), 1540(s), 1400(s), 1300(s). Four mL of 20% suspension (400 mg) of washed 175 nm carboxylate modified latex was diluted with 3 mL of ethoxyethanol in a 25 mL round bottom (R.B.) flask with a stir bar. The R.B. flask was then placed in an oil  
10 bath at 105°C and stirred for 10 minutes. Then, 3.3 mM C-28 thioxene and 15.5mM Eu(TTA)<sub>3</sub>DPP was added; the beads were stirred for 5 minutes more. At this point 1.0 mL of 0.1N NaOH was added slowly over 5 minutes. During all the additions, the oil bath temperature was maintained at 105°C. The oil bath temperature was slowly allowed to drop to room  
15 temperature over 2 hours. After cooling, the mixture was diluted with 20 mL of ethanol and centrifuged (12,500 rpm, 30 minutes). Supernatants were discarded and the pellets resuspended in ethanol by sonication. Centrifugation was repeated, and the pellet was resuspended in water; and centrifugation was repeated. The pellet was resuspended in 5 mL of  
20 aqueous ethanol to a final volume of 40 mL.

- Carboxyl acceptor beads prepared above (99mg in 4.5mL water) were added slowly with vortexing to 5.5mL of MAD aminodextran from above, followed by 1 mL of 200mg/mL NHS in 50 mM MES, pH 6, 1mL of 200 mg/mL EDAC in water, and 450  $\mu\text{L}$  of 1 M HCl, final pH 6. The  
25 mixture was incubated overnight at room temperature in the dark, then reacted with 200 mg succinic anhydride in 0.5 mL of DMSO for 30 min at room temperature. Freshly opened Surfact-Amps Tween-20 (Pierce Chemical Company, Rockford, Illinois) was added and the beads were centrifuged 30 min at 15,000 rpm in a Sorvall RC-5B centrifuge, washed by  
30 centrifugation with three 10mL portions of 50 mM MOPS, 50 mM EDTA,

0.1% Surfact-Amps Tween-20 (Pierce Chemical Company), pH 7.2, and resuspended in 3mL of the same.

Monoclonal anti-digoxin Ab (prepared as described above) was purified by ABx resin (Baker Chemical Company, Phillipsburg, NJ) and was  
5 dialyzed into 0.15 M NaCl, 5mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4. The anti-digoxin Ab was thiolated by mixing 622  $\mu$ L (4.28mg) with 10.2  $\mu$ L of SATA (1.25 mg/mL in ethanol, 2 eq.), incubating for 1 hr at room temperature and dialyzing cold against 2x2 L of 150 mM NaCl, 10mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, pH7. The thioacetylated antibody was deacetylated by adding 62.2  
10  $\mu$ L of hydroxylamine (1 M H<sub>2</sub>NOH, 50 mM MOPS, 25 mM EDTA, pH 7), bubbling with argon and incubating for 1 hr at room temperature. The product was applied to a Pharmacia PD-10 column (G-25) and eluted with 50 mM MOPS, 50 mM EDTA, pH 7.2, bubbled with argon. After 2.5 mL fore-run, three-1 mL fractions were collected and combined. Recovery of  
15 antibody was 3.66 mg or 86% by A<sub>280</sub>. Surfact-Amps Tween-20 (10%) was added to give 0.2% final concentration.

A 1.4 mL aliquot of the thiolated antibody above (1.71 mg antibody) was immediately added to 300  $\mu$ L (10 mg) of maleimidated beads prepared above plus enough 10% Tween-20 to bring final concentration of the  
20 mixture to 0.2%. The tube was purged with argon and incubated overnight at room temperature in the dark. To the above was added 3.4  $\mu$ L of 1 M HSCH<sub>2</sub>COOH in water. After 30 min at room temperature, 6.8  $\mu$ L of ICH<sub>2</sub>COOH (1 M in water) was added. After 30 min 3.5 mL of 0.17M glycine, 0.1M NaCl, 0.1% (v/v) Tween-20, 10 mg/mL BSA, pH 9.2 was  
25 added and the beads were centrifuged (30 min at 15,000 rpm), incubated for 3hr in 5 mL of the same buffer, centrifuged, washed by centrifugation with three-5 mL portions of Buffer C, resuspended in 5 mL of Buffer C and stored under refrigeration. The size of the beads, determined in Buffer C was 301 $\pm$ 56 nm. Binding capacity was determined with <sup>125</sup>I-digoxin and  
30 was equivalent to 377 antibody molecules per bead.

Silicon tetra-t-butyl phthalocyanine was prepared as follows:

Sodium metal, freshly cut (5.0g, 208mmol), was added to 300mL of anhydrous ether in a two-liter, 3-necked flask equipped with a magnetic stirrer, reflux condenser, a drying tube and a gas bubbler. After the sodium  
5 was completely dissolved, 4-t-butyl-1,2-dicyanobenzene (38.64g, 210mmol, from TCI Chemicals, Portland OR) was added using a funnel. The mixture became clear and the temperature increased to about 50°C. At this point a continuous stream of anhydrous ammonia gas was introduced through the glass bubbler into the reaction mixture for 1 hr. The  
10 reaction mixture was then heated under reflux for 4 hr. while the stream of ammonia gas continued. During the course of the reaction, as solid started to precipitate. The resulting suspension was evaporated to dryness (house vacuum) and the residue was suspended in water (400mL) and filtered. The solid was dried (60°C, house vacuum, P<sub>2</sub>O<sub>5</sub>). The yield of the product  
15 (1,3-diiminoisoindoline, 42.2g) was almost quantitative. This material was used for the next step without further purification. To a one-liter, three-necked flask equipped with a condenser and a drying tube was added the above product (18g, 89mmol) and quinoline (200mL, Aldrich Chemical Company, St. Louis MO). Silicon tetrachloride (11mL, 95mmol, Aldrich  
20 Chemical Company) was added with a syringe to the stirred solution over a period of 10 minutes. After the addition was completed, the reaction mixture was heated to 180-185°C in an oil bath for 1 hr. The reaction was allowed to cool to room temperature and concentrated HCl was carefully added to acidify the reaction mixture (pH 5-6). The dark brown reaction  
25 mixture was cooled and filtered. The solid was washed with 100mL of water and dried (house vacuum, 60°C, P<sub>2</sub>O<sub>5</sub>). The solid material was placed in a 1-liter, round bottom flask an concentrated sulfuric acid (500mL) was added with stirring. The mixture was stirred for 4 hr. at 60°C and was then carefully diluted with crushed ice (2000g). The resulting  
30 mixture was filtered and the solid was washed with 100mL of water and



- dried. The dark blue solid was transferred to a 1-liter, round bottom flask, concentrated ammonia (500mL) was added, and the mixture was heated and stirred under reflux for 2 hr., was cooled to room temperature and was filtered. The solid was washed with 50mL of water and dried under
- 5 vacuum (house vacuum, 60°C, P<sub>2</sub>O<sub>5</sub>) to give 12g of product silicon tetra-t-butyl phthalocyanine as a dark blue solid. 3-picoline (12g, from Aldrich Chemical Company), tri-n-butyl amine (anhydrous, 40mL) and tri-n-hexyl chlorosilane (11.5g) were added to 12g of the above product in a one-liter, three-necked flask, equipped with a magnetic stirrer and a reflux condenser.
- 10 The mixture was heated under reflux for 1.5 hr. and then cooled to room temperature. The picoline was distilled off under high vacuum (oil pump at about 1mm Hg) to dryness. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and purified using a silica gel column (hexane) to give 10g of pure product di-(tri-n-hexylsilyl)-silicon tetra-t-butyl phthalocyanine as a dark blue solid.
- 15 (MS: [M-H]<sup>+</sup> 1364.2, absorption spectra: methanol: 674nm (ε 180,000) : toluene 678nm, <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>): δ: -2.4(m, 12H), -1.3(m, 12H), 0.2-0.9 (m, 54H), 1.8(s, 36H), 8.3(d, 4H) and 9.6 (m, 8H) was consistent with the above expected product.
- 20 Sens-Sav - Sensitizer beads coupled to Streptavidin (2300 Sav/bead).
- The sensitizer beads were prepared placing 600mL of carboxylate modified beads (Seradyn) in a three-necked, round-bottom flask equipped with a mechanical stirrer, a glass stopper with a thermometer attached to it in one
- 25 neck, and a funnel in the opposite neck. The flask had been immersed in an oil bath maintained at 94+/-1°C. The beads were added to the flask through the funnel in the neck and the bead container was rinsed with 830mL of ethoxyethanol, 1700mL of ethylene glycol and 60mL of 0.1N NaOH and the rinse was added to the flask through the funnel. The funnel

was replaced with a 24-40 rubber septum. The beads were stirred at 765 rpm at a temperature of 94 $\pm$ 1°C for 40 min.

Silicon tetra-*t*-butyl phthalocyanine (10.0g) was dissolved in 300mL of benzyl alcohol at 60 $\pm$ 5°C and 85mL was added to the above round  
5 bottom flask through the septum by means of a syringe heated to 120 $\pm$ 10°C at a rate of 3mL per min. The remaining 85mL of the phthalocyanine solution was then added as described above. The syringe and flask originally containing the phthalocyanine was rinsed with 40mL of benzyl alcohol and transferred to round-bottom flask. After 15 min 900mL of  
10 deionized water and 75mL of 0.1N NaOH was added dropwise over 40 min. The temperature of the oil bath was allowed to drop slowly to 40 $\pm$ 10°C and stirring was then discontinued. The beads were then filtered through a 43 micron polyester filter and subjected to a Microgon tangential flow filtration apparatus (Microgon Inc., Laguna Hills, CA) using  
15 ethanol:water, 100:0 to 10:90, and then filtered through a 43 micron polyester filter.

Sulfo-SMCC (11.55mg) was dissolved in 0.5mL distilled water. Slowly, during 10 sec, the above solution was added to 5mL of stirring aminodextran (Molecular Probes, Eugene, Oregon) solution (12.5 mg/mL  
20 in 50mM MOPS, pH 7.2). The mixture was incubated for 1 hr at room temperature.

To the stirring solution above was added 5mL of 20mg/mL (100mg) of the sensitizer beads prepared above in distilled water. Then, 1mL of 200mg/mL NHS (prepared fresh in 50mM MES, pH adjusted to 6.0 with 6N  
25 NaOH). 200mg EDAC was dissolved in 1mL distilled water and this solution was added slowly with stirring to the sensitizer beads. The pH was adjusted to 6.0 by addition of 450 $\mu$ L of 1N HCl and the mixture was incubated overnight in the dark. A solution of 100mg of succinic anhydride in 0.5mL of DMSO was added to the sensitizer beads and the mixture was  
30 incubated for 30 min at room temperature in the dark. To this mixture was

added 0.13mL 10% Tween-20 bringing the final concentration of Tween-20 to 0.1%. The beads were centrifuged for 45 min at 15,000 rpm as above. The supernatant was discarded and the beads were resuspended in 10mL of buffer (50mM MOPS, 50mM EDTA and 0.1% Tween-20, pH 7.2). The mixture was sonicated to disperse the beads. The beads were centrifuged for 30 min as described above, the supernatant was discarded and the beads were resuspended. This procedure was repeated for a total of three times. Then, the beads were resuspended to 40mg/mL in 2.5mL of the above buffer, saturated with argon and Tween-20 was added to a concentration of 0.1%. The beads were stored at 4°C.

Streptavidin was bound to the above beads using 25mg streptavidin for 100mg of beads. 25mg streptavidin (50mg Aaston solid from Aaston, Wellesley, MA) was dissolved in 1mL of 1mM EDTA, pH 7.5, and 77 $\mu$ L of 2.5mg/mL SATA in ethanol was added thereto. The mixture was incubated for 30 min at room temperature. A deacetylation solution was prepared containing 1M hydroxylamine-HCl, 50mM Na<sub>2</sub>PO<sub>4</sub>, 25mM EDTA, pH 7.0. 0.1mL of this deacetylation solution was added to the above solution and incubated for 1 hr at room temperature. The resulting thiolated streptavidin was purified on a Pharmacia PD10 column and washed with a column buffer containing 50mM MOPS, 50mM EDTA, pH 7.2. The volume of the sample was brought to 2.5mL by adding 1.5mL of the above column buffer. The sample was loaded on the column and eluted with 3.5mL of the column buffer. The thiolated streptavidin was diluted to 5mL by adding 1.5mL of 50mM MOPS, 50mM EDTA, 0.1% Tween-20, pH 7.2. 5mL of the thiolated streptavidin solution was added to 5mL of the sensitizer beads, under argon, and mixed well. The beads were topped with argon for 1 min, the tube was sealed and the reaction mixture was incubated overnight at room temperature in the dark.

To the above beads was added 7.5mL of 50mM MOPS, 50mM EDTA, 0.1% Tween-20, pH 7.2 to bring the beads to 1mg/mL. The

remaining maleimides were capped by adding mercaptoacetic acid at a final concentration of 2mM. The mixture was incubated in the dark for 30 min at room temperature. The remaining thiols were capped by adding iodoacetic acid at a final concentration of 10mM and the mixture was  
5 incubated at room temperature for 30 min in the dark. The beads were centrifuged for 30 min at 15,000 rpm as above for a total of three times.

All oligonucleotides, including modified oligonucleotides, were from Oligos Etc., Wilsonville, OR.

Genomic DNA having the following point mutations within exon 11  
10 of the CFTR gene used herein:

Heterozygous DNA with one wild type (wt) allele and one of the following mutant alleles:

- G542X (G>T substitution) from Roche Molecular Systems, Alameda, California;
- 15 G551D (G>A substitution) from Roche Molecular Systems, Alameda, California;
- R553X (C>T substitution) from Roche Molecular Systems, Alameda, California;
- R560T (G>C substitution) from Roche Molecular Systems, Alameda,  
20 California.

#### Example 1

##### Study of Removal of Modified Nucleotides from the 3'-end of Oligonucleotide Primers

25 This experiment was carried out to demonstrate that (i) the presence of certain unnatural nucleotides at the 3'-end of an oligonucleotide primer prevents it from acting as a primer for amplification such as PCR and (ii) that the unnatural nucleotides can be removed by the 3'-5' exonuclease activity of thermostable Pfu polymerase thus activating the oligonucleotide

and allowing it to be extended by a polymerase and to function as a PCR primer.

The following oligonucleotides and modified oligonucleotides were employed in this example:

5 Oligonucleotide 5'GCCTTTCAAATTCAGATTGAGC 3' (P1)

(SEQ ID NO: 1)

Modified oligonucleotides 5'-GCCTTTCAAATTCAGATTGAGC-NN-G-3',

where N is:

- a) O-6-methyl deoxy G (P1a) (SEQ ID NO: 2)
- 10 b) O-4-methyl deoxy T (P1b) (SEQ ID NO: 3)
- c) etheno-deoxy A (P1c) (SEQ ID NO: 4)

The oligonucleotide (P1, P1a, P1b or P1c) was diluted to 1.25  $\mu$ M in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl<sub>2</sub>, 200  $\mu$ g/ml BSA (buffer A) and incubated with Pfu polymerase (0.625 U per 50  $\mu$ l) under conditions

15 mimicking PCR, i.e., incubated with polymerase but not target polynucleotide or dNTP's. Aliquots were withdrawn and chilled on ice after 10, 20, 30 and 40 cycles (30 sec at 94°C, 1 min at 64°C, 1 min at 72°C). Following this treatment the oligonucleotide was then used as a forward

20 primer in a PCR amplification to amplify a DNA fragment 203 bp in length corresponding to exon 11 of the human cystic fibrosis gene. The compositions of the lower and upper mixes were as follows:

<u>Lower mix</u>		<u>Upper mix</u>	
	Volume ( $\mu$ l)		Volume
25 (μl)			
H <sub>2</sub> O	8	H <sub>2</sub> O	17.25
10 X buffer A	2	10 X buffer A	2.5
Σ dNTP (250 $\mu$ M each)	4	Pfu (2.5 U/ $\mu$ l)	0.25
P1 (P1a, P1b, P1c) (1.25 $\mu$ M)	10	DNA	5
30 P2 (12.5 $\mu$ M)	1	(425 bp amplicon,	

1:1000)

An aliquot of a diluted amplicon 425 bp in length was used as a target polynucleotide. This amplicon was prepared using a WT genomic DNA (Roche Molecular Systems) as a target and primers P1' and P2' below. The not start procedure using PCR GEM50 wax beads (Perkin Elmer, Norwalk CT) was utilized. Wax beads were put in tubes containing the lower mix, the wax was melted and allowed to solidify, and the upper mix was then added. 20 PCR cycles were performed under the same conditions as above. 5 µl aliquots were analyzed in a 4-20 % gradient pre-cast gel (Novex, San Diego CA). The results obtained indicated that a certain delay in accumulation of the amplicon was observed for all three modified oligonucleotides. P1c appeared to be the most resistant to exonuclease cleavage. The time of delay that was estimated from the gel results was equivalent to less than 10 PCR cycles for P1a and P1b and about 30 PCR cycles for P1c.

All three 3'-modified oligonucleotides were then used in true PCR using genomic DNA. In that regard untreated oligonucleotides P1a, P1b and P1c were added to the reaction mixture. The observed delay in the appearance of the PCR product, as compared to P1, was shorter (equivalent to about 5 PCR cycles) and the difference between the three unnatural bases was much less pronounced, P1a being the least resistant to degradation by the Pfu polymerase.

### Example 2

#### PCR Using Oligonucleotide P1c

This experiment demonstrated that the use of 3'-etheno modified oligonucleotides (P1c and P2c) both as inner primers in nested PCR and by themselves greatly reduced the number of spurious amplification products as determined by gel electrophoresis.

In this experiment both forward (P1c above) and reverse (P2c below) primers contained 3'-etheno modified bases at their 3'-ends.

Primer P2: 5'-GACATTTACAGCAAATGCTTGC-3' (SEQ ID NO: 5)

Primer P2c: 5'-GACATTTACAGCAAATGCTTGC-NN-G-3', where N is  
5 etheno-deoxyA (SEQ ID NO: 6)

The outer primers in nested PCR were as follows:

P1' (forward): 5'-CAACTGTGGTTAAAGCAATAGTGT-3' (SEQ ID NO:7)

P2' (reverse): 5'-GCACAGATTCTGAGTAACCATAAT-3' (SEQ ID NO:8)

The anticipated product from primers P1' and P2' is a product 425 bp in  
10 length; the anticipated product from primers P1 and P2 is a product 203 bp  
in length. The 5'-end of P1 is located 53 nucleotides downstream from the  
5'-end of P1'.

In order to encourage non-specific priming (to demonstrate the  
advantages of the present invention), PCR cycling conditions were  
15 deliberately de-optimized by dropping the cycle annealing temperature  
(from 64°C as in Example 1 above to 50°C). For the same purpose, PCR  
was carried out without a hot start. Accordingly, all reaction components  
were mixed together at room temperature prior to carrying out the PCR  
reactions. 35 PCR cycles were performed using 50 ng genomic DNA and  
20 0.625 U Pfu polymerase per 50 µl.

The PCR products generated by using 3'-etheno modified  
oligonucleotides and ordinary or unmodified oligonucleotide primers of the  
same sequence were analyzed in a 4-20 % polyacrylamide gel (Novex).  
The results obtained are summarized as follows: When unmodified  
25 primers were used under suboptimal conditions, several non-specific  
products were observed in addition to the main band (a strong fast moving  
band(s) and several slowly moving weak bands). When the 3'-etheno  
modified oligonucleotides were used, the gel pattern cleared dramatically.  
The fast moving band and most of the slow moving parasite bands  
30 disappeared completely. A possible explanation is that the gradual

introduction of functional primers into the PCR reaction as the exonuclease activity of Pfu polymerase cleaves the modified nucleotides from their 3'-ends decreases the chances of false priming at the early stages of PCR, thus improving its overall specificity.

- 5           When Taq polymerase was used instead of Pfu, no amplification was observed. This demonstrated that the 3'-5' exonuclease activity present in Pfu polymerase, but absent in Taq polymerase, was responsible for the ultimate removal of unnatural etheno-modified nucleotides.

10

### Example 3

#### Mutation Detection Using Modified Oligonucleotides and Hot Start

Mutation detection was carried out in accordance with the disclosure in U.S. Patent Application Serial No. 60/009,289 filed December 22, 1995, *supra*, as described above.



PCR:

The hot start procedure using wax beads as described above in Example 1 was employed in this example. Thirty five PCR cycles (30 sec at 94°C, 1 min at 64°C, 1 min at 72°C) were performed using 50 ng genomic DNA and  
 5 0.625 U Pfu polymerase per 50 µl. The hot start procedure using PCR GEM50 wax beads (Perkin Elmer) was utilized. An equimolar mixture of the following primers (total concentration of each forward and reverse primers 250 nM) were used. The primers were chosen because they performed poorly in the mutation detection carried out without the use of  
 10 the present invention.

Unmodified primers:

P1-Bio: 5'GCCTTTCAAATTCAGATTGAGC 3' (SEQ ID NO: 1) (P1)  
 biotinylated at the 5'-end

P1-Dig: forward primer P1 labeled with digoxigenin at the 5'-end

15 P3: a reverse primer having a 3'-portion identical with P2 and an additional 5'-"tail" t1 (underlined) 20 nucleotides long:

5'-ACCATGCTCGAGATTACGAGGACATTTACAGCAAATGCTTGC-3'  
 (SEQ ID NO: 9)

P4: reverse primer having a 3'-portion identical with P2 and an additional  
 20 5'-"tail" t2 (underlined) 20 nucleotides long:

5'-GATCCTAGGCCTCACGTATTGACATTTACAGCAAATGCTTGC-3'  
 (SEQ ID NO: 10)

3'-etheno modified oligonucleotides (modified oligos) in accordance with the present invention:

25 P1c-Bio (P1c (SEQ ID NO: 4) biotinylated at the 5'end), P1c-Dig (P1c (SEQ ID NO: 4) labeled with digoxigenin at the 5'end), P3c (5'-ACCATGCTCGAGATTACGAGGACATTTACAGCAAATGCTTGC-NN-G-3' (SEQ ID NO: 11) where N is etheno-dA) and P4c (5'-GATCCTAGGCCTCACGTATTGACATTTACAGCAAATGCTTGC-NN-G-3'

(SEQ ID NO: 12) where N is etheno-dA) are the same as P1-Bio, P1-Dig, P3 and P4 but with two etheno-dA's followed by unmodified dG at their 3'-ends. These oligonucleotides were also purchased from Oligos, Etc.

Branch Migration:

- 5           After completion of PCR as described above, the samples were subjected to branch migration: 95°C, 1 min (denaturation), followed by 65°C, 30 min (branch migration).

Detection:

- Detection was carried out in a manner similar to that described in  
10 U.S. Patent No. 5,340,716, the relevant portions of which are incorporated herein by reference. Amounts of the components were as follows.

- A 2 µl aliquot of the above reaction mixture from the branch migration was mixed with 100 µl of the beads suspension (2.5 µg of Sens-Sav beads and 2.5 µg Acc-AbDig beads per 100 µl buffer A), incubated at  
15 37°C for 5 min. The reaction mixture was then irradiated with a 150 watt Xenon lamp for 3 sec (3 cycles of 1 sec illumination and 1 sec waiting time) and the signal was then read.

The results of a typical experiment conducted according to the protocol of this Example 3 were as follows (Table 1):

20

Table 1

Control

Invention

		<u>Unmodified primers</u>	<u>Modified</u>
<u>oligos</u>			
25	<u>Sample</u>	<u>Signal (RLU)</u>	<u>Signal</u>
	<u>(RLU)</u>		
	WT1	71252	6522
	WT2	75088	6186
	WT3	80016	7634
30	WT4	80052	6998

G542X/WT heterozygote	235644	576452
G551D/WT heterozygote	193542	426382
R553X/WT heterozygote	158058	390062
R560T/WT heterozygote	187078	440826

5

WT1-4 are wild type homozygotes.

The results obtained demonstrated that the use of 3'-etheno modified oligonucleotides in the branch migration assay resulted in significant improvement over the same assay using unmodified primers not in accordance with the present invention. The wild type signal decreased by an order of magnitude whereas the mutant signal increased by approximately a factor of two with the average signal-to-background ratio increasing from 2.5 (marginal) to 67.

15

#### Example 4

##### Mutation Detection Using Modified Oligonucleotides without Hot Start

In Example 3, the 3'-etheno modified oligonucleotides were aided by implementation of the standard hot start procedure utilizing wax beads. In this Example 4 the hot start procedure employed in Example 3 was not used. PCR conditions were the same as in Example 3, except that all components of the PCR reactions were mixed together at room temperature or on ice. The results are summarized in Table 2.

20

Table 2

	<u>Sample</u>	<u>Signal (RLU)</u>		<u>Signal (RLU)</u>	
		<u>RT</u>		<u>Ice</u>	
		<u>Control</u>	<u>Invention</u>	<u>Control</u>	<u>Invention</u>
		Unmodified primers	Modified oligos	Unmodified primers	Modified oligos
25					
30	WT1	97992	14988	112050	8612
	WT2	112110	10566	100784	9634

G551D/WT	59828	49910	99516	101016
R553X/WT	51640	45180	90592	121808

When the reactions were assembled at room temperature, the average  
 5 signal to background ratio of 5.3 was observed with the 3'-etheno modified  
 oligonucleotides as compared to no discrimination between mutant and  
 wild type samples with the unmodified primers (Table 2, left). When the  
 reactions were assembled on ice, the average signal to background ratio  
 increased to 24.2 for the 3'-etheno primers, whereas still no discrimination  
 10 was observed for the unmodified primers (Table 2, right).

The above results demonstrated that the 3'-5' exonuclease activity  
 of the Pfu polymerase was occurring at room temperature and removed the  
 modified nucleotides from some of the molecules of the modified  
 oligonucleotides before PCR cycling began. To slow this process down,  
 15 smaller amounts of Pfu polymerase were used. In order to preserve the  
 efficiency and yield of PCR, Pfu polymerase was supplemented with Pfu  
 exo<sup>-</sup> (from Stratagene, La Jolla CA) in which the 3'-5' exonuclease activity  
 is missing. PCR conditions were the same as above, except that 40 cycles  
 were performed. The total amount of polymerase (Pfu + Pfu exo<sup>-</sup>) per  
 20 reaction was 0.625 U. To make the conditions maximally unfavorable (to  
 encourage non-specific priming), PCR reactions containing 3'-etheno  
 modified oligonucleotides were left at RT for as long as 30 min prior to  
 starting thermocycling. The results are summarized in Table 3.

25

Table 3Signal (RLU)

<u>Sample</u>	<u>Pfu</u>	<u>Pfu exo<sup>-</sup>/Pfu</u> (2:1)	<u>Pfu exo<sup>-</sup>/Pfu</u> (1:1)
30 WT1	18970	8704	14540

WT2	18652	9234	21656
G551D/WT	103236	173964	314368
R553X/WT	75130	210706	337192

5 The results summarized in Table 3 demonstrated that, when the mixture of two enzymes (Pfu and Pfu exo<sup>+</sup>) was used, the 3'-etheno primers were very effective as judged by a signal to background ratio of about 20 as compared with a signal to background ratio of about 4.5 for Pfu alone.

10 By employing the method of the present invention in conjunction with the mutation detection, a substantial improvement of the signal-to-background ratio in a direct approach as applied to the detection of 4 point mutations in exon 11 of the human CFTR gene was realized as demonstrated by this Example 5. The approach of the present invention as applied to the above mutation detection method achieves at least the  
15 same results as the known hot start method using wax beads.

The above discussion includes certain theories as to mechanisms involved in the present invention. These theories should not be construed to limit the present invention in any way, since it has been demonstrated that the present invention achieves the results described.

20 The above description and examples fully disclose the invention including preferred embodiments thereof. Modifications of the methods described that are obvious to those of ordinary skill in the art such as molecular biology and related sciences are intended to be within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for selectively extending an oligonucleotide primer  
along a specific target polynucleotide sequence in a mixture of  
5 polynucleotides, said method comprising:
  - (a) providing in combination said mixture and a modified  
oligonucleotide having a 3'-end that is inefficiently extendable along any  
polynucleotide sequence and
  - (b) extending said oligonucleotide primer along said specific  
10 target polynucleotide sequence by controlling the degradation of said 3'-  
end of said modified oligonucleotide thereby producing said  
oligonucleotide primer.
2. The method of Claim 1 wherein said degradation is  
15 enzymatically catalyzed.
3. The method of Claim 1 wherein said modified  
oligonucleotide comprises one or more nucleotide analogs at said 3'-end  
that inhibit chain extension of said modified oligonucleotide along all  
20 polynucleotides in said mixture.
4. The method of Claim 3 wherein said one or more nucleotide  
analogous hybridize inefficiently to any normal nucleotide in a  
polynucleotide.  
25
5. In a method for amplifying a target polynucleotide sequence,  
said method comprising combining said target polynucleotide sequence  
with reagents for amplifying said target polynucleotide sequence and  
subjecting said combination to conditions wherein said target  
30 polynucleotide sequence is amplified, said reagents comprising an

oligonucleotide primer and a polymerase, the improvement which comprises said oligonucleotide primer being derived from a modified oligonucleotide having a portion that hybridizes to said target polynucleotide sequence except for the 3'-end thereof, said 3'-end  
5 having at least one nucleotide analog that is substantially incapable of hybridizing to a polynucleotide.

6. The method of Claim 5 wherein said modified oligonucleotide has a 3'-end that comprises at least two unnatural  
10 nucleotides that do not hybridize to said target polynucleotide sequence.

7. The method of Claim 1 wherein said nucleotide analog is a natural nucleotide having a chemical modification that is removable.

15 8. The method of Claim 5 wherein said nucleotide analog is removable by an enzyme having 3' to 5' exonuclease activity.

9. The method of Claim 7 wherein said chemical modification is selected from the group consisting of esters, glycosides, sulfates and  
20 amides.

10. A method for amplifying a target polynucleotide sequence, which comprises:

(a) providing in combination (i) said target polynucleotide  
25 sequence and (ii) reagents for conducting an amplification of said target polynucleotide sequence, said reagents comprising a polymerase, nucleoside triphosphates and a modified oligonucleotide comprising at least one nucleotide analog at the 3'-end thereof, said nucleotide analog being substantially non-hybridizable to any polynucleotide sequence and

being removable by a 3' to 5' exonuclease at a rate that is slower than for a natural nucleotide,

- (b) subjecting said combination to conditions for amplifying said target polynucleotide sequence wherein said nucleotide analog is removed from said modified oligonucleotide to give an oligonucleotide primer, which is then extended along said target polynucleotide sequence to produce an extended oligonucleotide primer.

11. The method of Claim 10 which comprises detecting said target polynucleotide sequence by detecting said extended oligonucleotide primer.

12. The method of Claim 10 wherein said reagents comprise a second modified oligonucleotide that is degradable to a second oligonucleotide primer.

13. The method of Claim 10 wherein said nucleotide analog has a 3'-hydroxyl group.

14. The method of Claim 10 wherein said nucleotide analog is a natural nucleotide having a chemical modification that is removable.

15. The method of Claim 10 wherein said nucleotide analog is removable by an enzyme having 3' to 5' exonuclease activity.

16. The method of Claim 14 wherein said chemical modification is selected from the group consisting of esters, glycosides, sulfates and amides.



17. The method of Claim 10 wherein said nucleotide analog is at the 3'-terminus of said modified oligonucleotide.

18. The method of Claim 10 wherein said nucleotide analog is  
5 within one nucleotide of the 3'-terminus of said modified oligonucleotide.

19. The method of Claim 10 wherein said nucleotide analog is attached directly to a sequence of 12 to 50 nucleotides of said modified oligonucleotide that hybridize to said target polynucleotide sequence.  
10

20. The method of Claim 10 wherein said nucleotide analog is comprised of an unnatural base selected from the group consisting of adenine modified at the 6-amino group, guanine modified at the 6-oxygen, thymine modified at the 4-oxygen, purines modified at the ring nitrogens at  
15 the 5-position, pyrimidines modified at the ring nitrogens at the 3-position, guanine modified by removal of the 2-amino group and cytosine modified by the removal of the 4-amino group.

21. The method of Claim 10 wherein said target polynucleotide  
20 is DNA.

22. In a method for amplifying a target polynucleotide sequence, said method comprising combining said target polynucleotide sequence with reagents for amplifying said target polynucleotide sequence and  
25 subjecting said combination to conditions wherein said target polynucleotide sequence is amplified, said reagents comprising an oligonucleotide primer and a polymerase, the improvement which comprises said oligonucleotide primer being derived from a modified oligonucleotide having a portion that hybridizes to said target  
30 polynucleotide sequence except for the 3'-end thereof, said 3'-end being

degradable at a rate of degradation of said oligonucleotide primer hybridized to said target polynucleotide sequence is slow relative to the rate of degradation of said 3'-end of non-hybridized oligonucleotide primer.

5

23. In a method for forming multiple copies of a target polynucleotide sequence, said method comprising:

(a) hybridizing to the 3'-end of said target sequence a first oligonucleotide primer ("first primer"),

10

(b) extending, in the presence of a polymerase, said first primer along at least said target sequence, said first primer being capable of hybridizing to, and being extended along, (1) said extended first primer or (2) an extended second oligonucleotide primer ("second primer") wherein said extended second primer results from the extension of a second primer capable of hybridizing to and extending along a polynucleotide that is complementary (complementary polynucleotide) to said target sequence,

15

(c) dissociating said extended first primer from said target sequence,

20

(d) hybridizing, to the 3'-end of said extended first primer, said first or said second primer,

(e) extending said first or said second primer along said extended first primer,

25

(f) dissociating said extended first primer or said extended second primer from said extended first primer,

(g) hybridizing, to the 3'-end of said extended first or second primer, said first primer, and

(h) repeating steps (e)-(g),

the improvement comprising said first oligonucleotide primer being

30

derived from a modified oligonucleotide having a portion that hybridizes

to said target polynucleotide sequence except for the 3'-end thereof, said 3'-end having at least one nucleotide analog that is incapable of hybridizing to a polynucleotide.

5           24.    The method of Claim 23 wherein said modified oligonucleotide has a 3'-end that comprises at least two unnatural nucleotides that do not hybridize to said target polynucleotide sequence.

          25.    The method of Claim 23 wherein said nucleotide analog is a  
10   natural nucleotide having a chemical modification that is removable.

          26.    The method of Claim 23 wherein said nucleotide analog is removable by an enzyme having 3' to 5' exonuclease activity.

15           27.    The method of Claim 25 wherein said chemical modification is selected from the group consisting of esters, glycosides, sulfates and amides.

          28.    The method of Claim 23 wherein the presence of said  
20   extended first primer and/or said extended second primer is detected and related to the presence of said target polynucleotide.

          29.    The method of Claim 23 wherein the repeating of steps (e)-  
25   (g) is achieved by repeated temperature cycling.

          30.    The method of Claim 29 wherein temperature cycling is repeated at least 3 times.

          31.    The method of Claim 23 wherein said target polynucleotide  
30   is DNA.

32. The method of Claim 23 wherein said extending is carried out in the presence of nucleoside triphosphates.

5

33. The method of Claim 23 wherein said first and said second primers are different.

34. The method of Claim 23 wherein said first and said second primers are different and said extended first primer is a template for said second primer and said extended second primer is a template for said first primer.

35. A kit comprising in packaged combination:  
(a) a modified oligonucleotide comprising at least one nucleotide analog at the 3'-end thereof, said nucleotide analog being substantially non-hybridizable to any normal nucleotide in a polynucleotide sequence,

15

(b) nucleoside triphosphates, and  
(c) an enzyme having 3' to 5' exonuclease activity.

20

36. The kit of Claim 35 comprising a second modified oligonucleotide.

37. The kit of Claim 37 wherein said nucleotide analog has a 3'-hydroxyl group.

25

38. The kit of Claim 35 wherein said nucleotide analog is a natural nucleotide having a chemical modification that is removable.

30

39. The kit of Claim 35 wherein said nucleotide analog is removable by an enzyme having 3' to 5' exonuclease activity at a rate that is slower than for a natural nucleotide.

40. The kit of Claim 38 wherein said chemical modification is  
5 selected from the group consisting of esters, glycosides, sulfates and amides.

41. The kit of Claim 35 wherein said nucleotide analog is selected from the group consisting of adenine modified at the 6-amino group,  
10 guanine modified at the 6-oxygen, thymine modified at the 4-oxygen, purines modified at the ring nitrogens at the 5-position, pyrimidines modified at the ring nitrogens at the 3-position, guanine modified by removal of the 2-amino group and cytosine modified by the removal of the 4-amino group.

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42. A method for controlling the concentration of an oligonucleotide primer in a reaction medium for conducting a reaction in which said oligonucleotide primer is extended along a polynucleotide template, said method comprising:

20 (a) providing in said reaction medium, together with reactants for extending said oligonucleotide primer along said polynucleotide template, a modified oligonucleotide having a degradable 3'-end that is substantially incapable of being extended along said polynucleotide template, the degradation thereof forming said oligonucleotide primer  
25 and

(b) subjecting said reaction medium to controlled conditions for degrading said 3'-end of said modified oligonucleotide thereby forming said oligonucleotide primer.

43. The method of Claim 42 wherein said 3'-end comprises at least one nucleotide analog that is incapable of hybridizing with a natural nucleotide.

\* \* \* \* \*

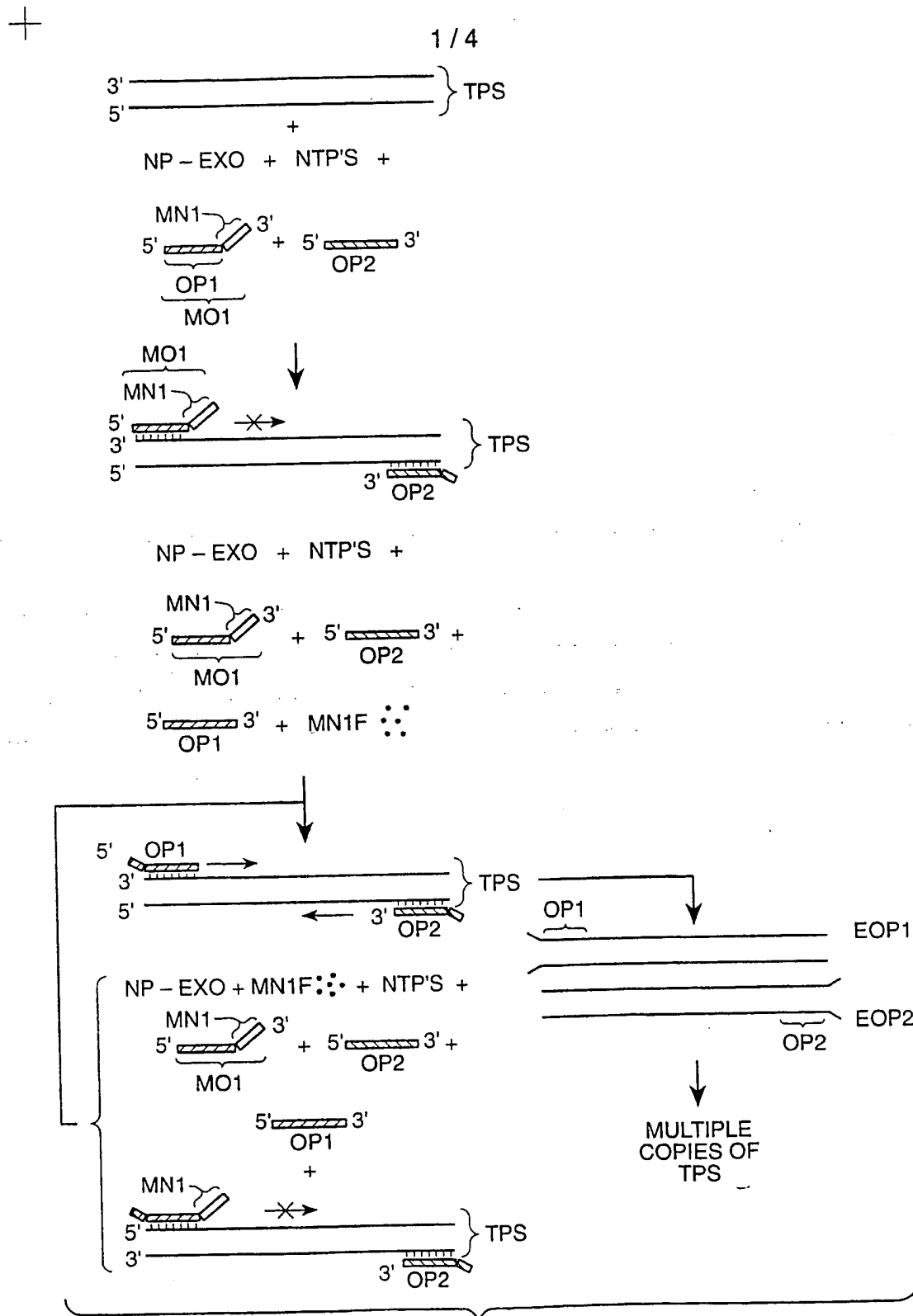


FIG. 1





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**FIG. 3**

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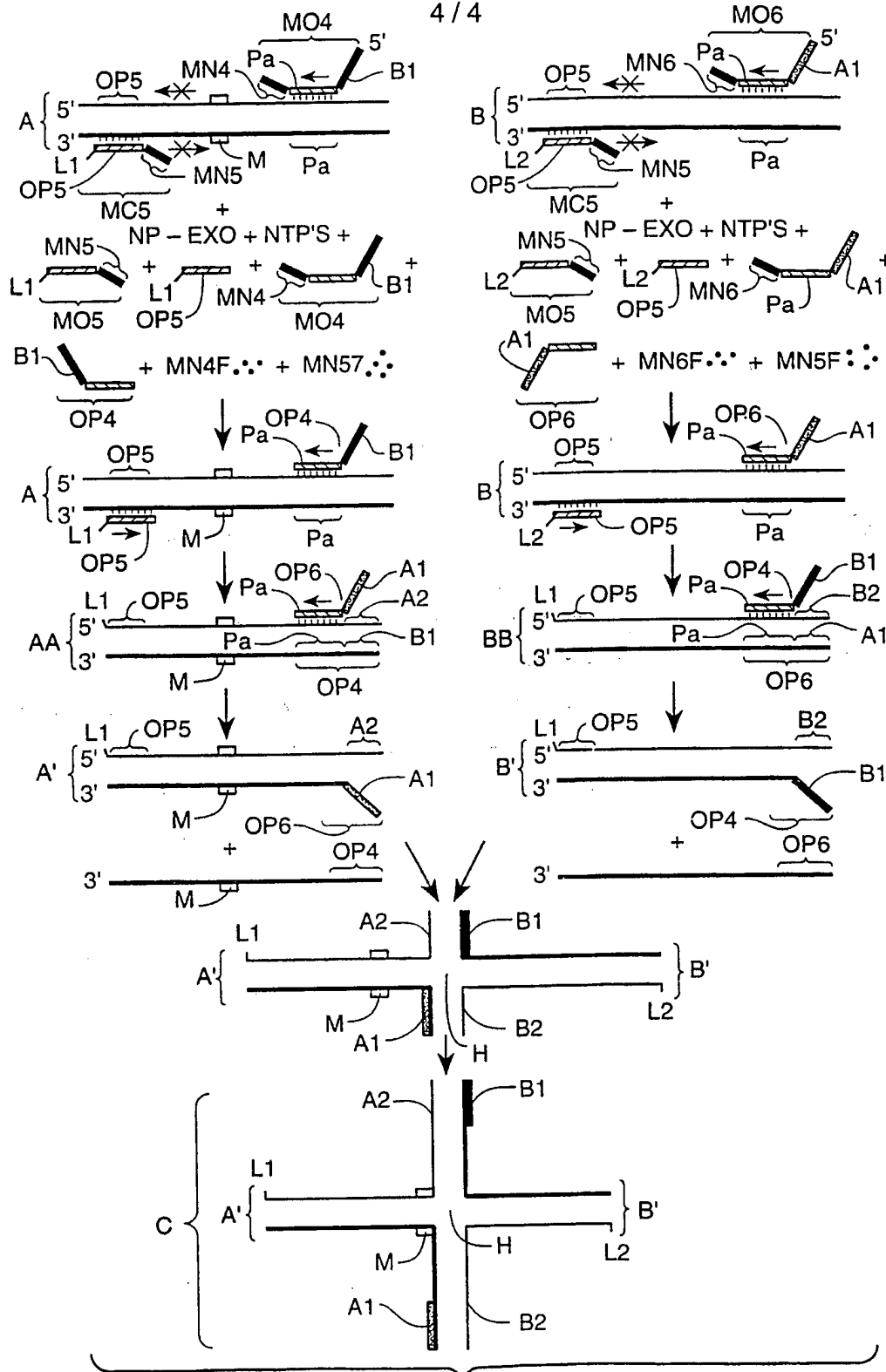


FIG. 4

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 97/23706

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C12Q1/68		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12Q		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 549 107 A (SYNTEX INC) 30 June 1993  * see especially the claims * see the whole document ---	1-19, 21-40, 42, 43
X	EP 0 744 470 A (JOHNSON & JOHNSON CLIN DIAG) 27 November 1996  see the whole document ---  -/---	1-19, 21-40, 42, 43
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
<b>* Special categories of cited documents :</b>		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search  <div style="text-align: center;">9 June 1998</div>		Date of mailing of the international search report  <div style="text-align: center;">17/06/1998</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016		Authorized officer  <div style="text-align: center;">Knehr, M</div>

# INTERNATIONAL SEARCH REPORT

Inter: onal Application No  
PCT/US 97/23706

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NORONHA DE C M C ET AL: "AMPLIMERS WITH 3'-TERMINAL PHOSPHOROTHIOATE LINKAGES RESIST DEGRADATION BY VENT POLYMERASE AND REDUCE TAQ POLYMERASE MISPRIMING" PCR METHODS AND APPLICATIONS, vol. 2, no. 2, November 1992, pages 131-136, XP000600686 see the whole document ---	1-19, 21-34, 42,43
X	SKERRA A: "PHOSPHOROTHIOATE PRIMERS IMPROVE THE AMPLIFICATION OF DNA SEQUENCES BY DNA POLYMERASES WITH PROOFREADING ACTIVITY" NUCLEIC ACIDS RESEARCH, vol. 20, no. 14, 25 July 1992, pages 3551-3554, XP000651211 see the whole document ---	1-19, 21-34, 42,43
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Y	WO 96 02672 A (BEHRINGWERKE AG ;ULLMAN EDWIN F (US)) 1 February 1996 see abstract see claims 4-7 ---	1-34,42, 43
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P,X	WO 97 23646 A (BEHRINGWERKE AG ;ULLMAN EDWIN F (US)) 3 July 1997 see abstract see page 4, line 14 - line 27 see page 15, line 34 - line 40; claims 1-11; figure 3; examples 12,13 -----	1-43

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